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**FUNCTION OF SINGLE-STRANDED DNA-BINDING
PROTEIN OF BACTERIOPHAGE T7**

KIKUO SHIMIZU

1990

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Preface

Bacteriophage T7 is one of the phage studied extensively, and the mechanism of its DNA replication and recombination is well known.

Genetic cross-experiments of bacteriophage T7 suggested that products of the genes 2.5, 3, 4, 5, and 6 are required for recombination as well as DNA replication (1-3). To elucidate roles of these products in recombination, isolation of the intermediate molecules of genetic recombination was carried out from the T7-infected cells under the absence of each of these functions (4, 5). Based on these results, roles of the relevant gene products were hypothesized as follows. Gene 6 product, 5'-exonuclease, makes gaps from nicks, which have been introduced at random or specifically. Gapped molecules can pair each other with their complimentary gapped regions and form a doubly branched molecule with letter H structure. In these processes, single-stranded DNA-binding protein (ssb, the product of the gene 2.5) is involved and stimulates formation of the doubly branched molecules. Gene 3 product, endonuclease, processes the branches to form a linear recombinant molecule. T7 DNA polymerase, specified by the gene 5, stimulates formation of the branched molecules by its exonucleolytic activity and/or repair activity by a single strand-replacing synthesis.

T7 ssb plays also an important role in DNA replication *in vitro*. It stimulates the polymerization activity of T7 DNA polymerase on single stranded templates, and also stimulates the exonuclease activity of T7 DNA polymerase on a duplex DNA template (6,7). T7 5'-exonuclease activity of the gene 6 product was also stimulated by T7 ssb (8). *In vitro*, *E.coli* ssb protein could substitute for T7 ssb with regard to its stimulatory effect on the activity of T7 DNA polymerase (9). This result suggested that T7 ssb was not essential for T7 phage growth, but could be replaced by *E.coli* ssb.

Both replication and recombination of DNA are important for the transfer of genetic information, and T7 ssb participates in both process, and it is believed to interact with several proteins which participate in replication and recombination.

In this work, I will describe the isolation and characterization of a temperature sensitive ssb mutant and amber mutants, and will show that T7 ssb is essential for the phage growth. I will also present the evidence that T7 ssb interacts with other T7 coded proteins, exonuclease (gene 6 product) and gene 18 product.

The regulation of expression regulation of the gene 2.5 operon was also suggested.

Kikuo Shimizu

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CHAPTER I

Isolation and characterization of the gene 2.5 mutants

Abstract

We isolated three new mutants in the gene 2.5 which encodes a single-stranded DNA binding protein, ssb (232 amino acids) of bacteriophage T7. One is a temperature sensitive mutant *ts2.5*, which was isolated after mutagenization with NTG in the phage infected cells. The others are amber mutants (*am2.5-1* and *am2.5-2*) whose mutations were introduced by *in vitro* mutagenesis.

The *am2.5-1* mutation was designed to encode a 15 amino acids polypeptide and *am2.5-2* to encode a 200 amino acids polypeptide.

All these mutants could not make viable progeny under the restrictive condition, at 42°C or in suppressor free host bacteria even if the bacterial had the wild type ssb gene.

These results, however, rendered us a suspicion against T7 *up2* mutant, whose growth was dependent the host ssb (Araki and Ogawa,1981).

The analysis of T7 *up2* revealed that the mutation in the gene 2.5 of T7 *up2* was an opal mutation, *opl*, and this mutant gene encoded a protein lacking 17 amino acid residues from C-terminal.

Moreover we found that T7 *up2* mutant had two other mutations outside the ssb gene, that enabled T7 phage to use bacterial ssb.

The locations of these mutations on T7 phage genome were roughly determined by replacing each part of the mutant DNA by the corresponding wild type DNAs *in vitro*. Their precise locations were then determined by the genetic cross experiments. One mutation was found in the gene 6 and the other was in the gene 18. Those two mutations had no phenotype by themselves. Moreover, the phage that has *opl* mutation alone (T7 *opl*) could not grow on *su*⁻ strain even if the host strain had the *ssb*⁺ gene.

The recombination and DNA synthesizing abilities of T7 ssb mutants were measured. The both abilities of T7 *opl*, *am2.5-1* and *am2.5-2* phages were reduced on nonpermissive host as expected from the results of T7 *up2* phage, but T7 *ts2.5* synthesized DNA at nonpermissive temperature unexpectedly. The synthesized DNA in

T7 *ts2.5* infected cells at nonpermissive temperature were revealed to be concatemeric DNA by the density gradient sedimentation analysis.

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Introduction

A single-stranded DNA-binding protein (ssb) exists universally in living organisms (Stephen C *et al.* 1981). These proteins are generally a single-chain protein with a molecular weight close to 30,000. In spite of a small size protein, ssb takes is involved in DNA replication and recombination in *E.coli* and bacteriophage (Morrical, Lee and Cox 1986). The ssb of bacteriophage T7 plays an important role for its DNA replication and recombination. In replication, ssb stabilizes the single stranded region of DNA during lagging strand replication (Delius, Mantell and Alberts 1972). In recombination, ssb enhances the activity of 5'-exonuclease (gene 6 product)(Sadowski 1984).

The mutant of ssb of bacteriophage T7 had not been available recently although the ssb protein of bacteriophage T7 was purified. It was thought that one of the reason why the mutant had not been available was substitution of ssb defect in T7 phage with the bacterial ssb. Actually, this kind of substitution was observed for the mutations in the gene 1.3 (ligase) and the gene 1.2 (replication factor). T7 ligase mutant was not isolated until ligase deficient bacterial strain was used for (Masamune, Frankel and Richardson 1971), and *E.coli optA* product was found to be able to substitute for the phage gene 1.2 protein (Saito, Tarbor, Tamanoi and Richardson 1980; Saito and Richardson 1981).

Based on the above consideration, the mutant of ssb of T7 phage, *up2* was isolated by using *E. coli* ssb deficient strain at our laboratory , and named as the gene 2.5, since it was between the gene 2 and the gene 3 (Araki and Ogawa 1981a), and Studier isolated T7 ssb mutant, *J27a* by the gel electrophoresis for altered T7 ssb protein (1981).

In this paper, we will describe the characteristics of T7 *up2* and findings that T7 *up2* strain bears three mutations: an opal mutation in the gene 2.5 and two other mutations in the gene 6 and the gene 18. These two mutations outside the gene 2.5 allow the mutant to grow on *E. coli* sup⁺ ssb⁺ strain when the product of gene 2.5 was deficient. Moreover we isolated the new mutants of ssb which have no activity at non-permissive

condition and characterized them to consider the roles of ssb gene *in vivo*. The results show that T7 ssb protein is necessary to DNA replication, recombination and repair, and that *E.coli* ssb cannot substitute for phage ssb *in vivo*.

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Materials and Methods

Buffers and Media:

T-broth contains 1% Bactotryptone (Difco) and 0.25% (w/v) NaCl.

Cas- λ broth contains 1% Casamino Acids (Difco, vitamin-free), 0.55 NaCl and 1 μ g/ml vitamin B1 (pH 7.0). M9 medium was described previously (Tsujimoto and Ogawa 1977). T7 buffer contains 10 mM Tris-HCl (pH 7.4), 1 mM MgSO₄, 5% NaCl and 10 mg/ml gelatin. TEN buffer contains 10 mM Tris-HCl (pH 7.4), 1 mM EDTA and 10 mM NaCl.

SSC contains 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0). All of radioisotopes were purchased from Amersham, Japan.

Enzymes and Chemicals:

Restriction enzyme *Eco*RI was prepared as described by Wilson and Young (1975). Restriction enzyme *Bgl*II, *Bcl*II, *Bst*EII, and *Xba*I were purchased from Takara-Shuzo, Japan, and New England Biolab, U.S.A.. T4 DNA ligase was purified as described by Weiss *et al.* (1968). T4 polynucleotide kinase was purified as described by Richardson C. C. (1965). Low melting temperature agarose was purchased from BioRad Lab, U.S.A. BU was purchased from YAMASA. Oligonucleotide-directed mutagenesis system (ver.2) was from Amersham. T7 sequencing kit was from Pharmacia.

DNA and Oligonucleotides:

M13mp18 DNA was purchased from Takara. The 36 mer (5'-GGGTACCGCTGA-ACCTTAGGCTTACATCGCCAAGC-3') for introducing *am2.5-1* mutation was synthesized by DNA synthesizer (Applied Biosystems) in our laboratory, and two 17 bp oligonucleoties, (5'-GAGGTTACTCGTGTGCG-3') for DNA sequencing, (5'-ATGTTGCCTAGGGTTCTGCC-3') for introducing *am2.5-2* mutation were purchased from Takara.

Bacterial and Phage strains:

Bacterial strains and T7 phage strains used in this CHAPTER were described in Table 1. and Table 2. respectively. T7 *op1* mutation in the gene 2.5 was separated from up2 (Araki and Ogawa 1981a). T7 amber mutants were obtained from Dr. F. Studier (1969).

Isolation of mutants:

T7 wild type phage was mutagenized by following procedure.

E.coli B, 011' was grown to 3×10^8 cells/ml in T-broth supplemented with 1 mM MgSO₄. To the culture NTG*¹ was added to a final concentration of 200 µg/ml and the bacterial cells were infected with T7 wild type phage at a multiplicity of 0.1. The mixture was incubated until complete lysis occurred. After complete lysis occurred, the lysate was centrifuged at 8,000 rpm for 10 min at 4°C to remove debris and the supernatant was recentrifuged to remove residual NTG at 36,000 rpm for 1 hr at 4°C in Beckman sw 50.1 rotor. The pellet was suspended in T7 buffer.

Mutagenized phages were plated with an overnight culture of 011' at 32°C.

Cloning of T7 gene 2.5 region:

The *Bcl*I-*Bgl*II region (3120bp) of T7 DNAs were cloned into the *Bam*HI site of M13mp18 DNA for DNA sequencing and oligonucleotide-directed mutagenesis.

The *Bcl*I-*Bgl*II region includes the genes 2-3.5.

Sequencing of mutant DNA:

Sequencing of mutant DNA was carried out based on M13 method using Pharmacia T7 sequencing kit. Method of cloning of the fragment containing the gene 2.5 mutations was described in the text.

Oligonucleotide-directed mutagenesis:

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Mutagenesis was carried out by Amersham oligonucleotide- directed mutagenesis system (ver 2).

Transfer of Mutations into T7 phage:

The gene 2.5 mutations introduced by the site directed mutagenesis were transferred from M13 phage DNA into T7 phage DNA by *in vitro* recombination. T7 DNA was digested by restriction enzymes *Tth111I* which cuts at the 8,894th base and *BglII* which cuts at the 11,515th base. M13 phage DNA was digested by restriction enzymes *Tth111I* and *Sau3A*. The digested M13 DNA was applied to agarose gel electrophoresis and 2,622bp fragment was isolated from the agarose. Digested T7 DNA and 2,622bp M13 DNA fragment were mixed and ligated by Takara ligation kit. *E.coli* C600^{r⁻m⁻recB was transfected with the ligated DNA by the standard method.}

The plaques appeared were picked up and analyzed the plating efficiency on various hosts. T7 DNA was isolated and screened for appropriate restriction sites.

UV irradiation:

Phage stocks were diluted to 10⁹ phages/ml in T7 buffer and irradiated in a glass petri dish with constant agitation under a 15-watt low-pressure germicidal lamp at a dose rate about 1.0 J m⁻² sec⁻¹. Irradiated samples were plated and incubated under the dark to avoid photoreactivation.

Analysis of mutant proteins of ssb:

Bacterial strain 594 was grown to the density of 3 x 10⁸ cells/ml in M9 medium supplemented with 0.2% Glucose and 0.2% Casamino Acid (Difco). The culture (5 ml) was irradiated by UV light at a dose of 1000 J/m². After culturing further for 30 min, the cells were infected with T7 phage at a multiplicity of 20. After 5 min of infection, [³⁵S]-Methionine (43.8 GBq/μmol) was added to the cultures at an specific activity, 37 KBq/ml. After 15 min of incubation, the infected cells were collected by centrifugation at 8000 rpm at 4°C for 10 min. The pellet was suspended in 0.1 ml of

SDS buffer, and was heated at 95°C for 5 min. The sample was subjected to SDS gel electrophoresis by Laemmli's method (Laemmli 1970).

Measurement of DNA synthesis:

Bacterial cells were grown to the density of 3×10^8 cells/ml in M9 medium supplemented with 0.2% Glucose, 0.2% Casamino Acids, 2 μ g/ml thymine, and irradiated with UV at a dose of 500 J/m². After incubation at 37°C for 30 min further [³H]-thymidine (1.67 GBq/ μ mol) was added to the culture at 37 KBq/ml. After 5 min, phages were added to each culture at a multiplicity of 10. At a time indicated, 100 μ l of samples were removed and added with the equal volume of 10% TCA*² and chilled on ice, and their acid-insoluble radioactivities were determined.

Preparation of labeled phages:

[³²P]-labeled or [³H]-labeled T7 phage was prepared as described previously (Shimizu, Araki and Ogawa, 1985) except that bacterial host 011' was used instead of C600thyA. [³H]-BU-labeled phage was prepared by the following procedure. 011' cells were grown to 1×10^8 cells/ml in Cas- λ broth supplemented with 4 μ g thymine/ml at 37°C, and harvested by centrifugation at room temperature and resuspended in Cas- λ broth supplemented with 10 lg BU*³, 1 μ g thymine, and 37 KBq [³H]-thymidine (1.67 GBq/ μ mol) per ml. After culturing for 90 min, cells were infected with T7 phage at a multiplicity of 0.1. After complete lysis occurred, phages were purified by differential centrifugations and banding in a CsCl density gradient.

Isolation of intermediate molecules in recombination:

Bacterial strain 594 was grown to the density of 3×10^8 cells/ml in T-broth supplemented with 1 mM MgSO₄ at 32°C or 42°C. [³²P]-labeled and [³H]-BU labeled phage was added to the culture at a multiplicity of 10 each. After adsorption for 5 min, the infected cells were shaken for 10 min and collected by centrifugation by 8,000 rpm at 4°C. The pellets were suspended in SSC with 100 mg/ml lysozyme. The solutions

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were treated with 3 cycles of freezing and thawing in dry ice/acetone bath, and added with Pronase and SLS*4 at a concentration of 1 mg/ml and 1%, respectively. The mixtures were incubated at 37°C for 1 hr, and then followed with CsCl density gradient centrifugation.

Analysis of intracellular DNA in T7 phage-infected cells:

E.coli 594 was grown at 30°C to 3×10^8 cells/ml in M9 medium. The culture was divided into 4 parts. Each portion was infected with one of the following phage; T7 wild type, T7 *opI*, T7 *opIS-1*, and T7 *opIS-2* at a multiplicity of infection of 10. At 8 min after infection [^3H]-thymidine (1.67 GBq/ μmol) was added to each infected culture at 37 KBq/ml. At 10 min after infection samples were ice chilled with TEN-buffer. The DNA was extracted by the method described previously (Shimizu, Araki and Ogawa 1985) and analyzed by neutral sucrose gradient sedimentation with [^{32}P]-labeled marker T7 DNA (Miller and Marion 1976). After centrifugation the sample was collected from the bottom of a tube and acid-insoluble radioactivity was determined. Almost all [^3H]-labeled DNA was found in the newly synthesized T7 DNA which was identified by DNA-DNA hybridization (Miller and Marion 1976). The [^{32}P]-labeled marker DNA was extracted from [^{32}P]-labeled T7 phage particles.

Extraction of Phage DNA:

Purification of phage and preparation of phage DNA was described previously (Shimizu, Araki and Ogawa 1985).

Genetic cross of T7 phages:

One of fresh culture of *E.coli* grown in T-broth supplemented with 1 mM MgSO_4 to 2×10^8 cells/ml was added with freshly prepared parental T7 phages at a multiplicity of infection of 10 each.

The infected cells was kept standing at 37°C for 5 min, and then treated with T7 phage specific antiserum at a final K value of 5 for 5 min at 37°C. The infected cells were diluted 1:10³ with T-broth and the number of infective centers/ml was measured.

The diluted infected cells were was incubated at 37°C for 1 hr. to allow phage growth. The recombination frequency was deduced by calculating from the ratio of number of progeny phages on non-permissive host to those on permissive host.

Transfection of bacterial cells by T7 phage DNA:

Transfection of bacterial cells by T7 DNA was carried out by the method of Cameron *et al.*(1975).

Construction of recombinant T7 DNA:

T7 *up2* DNA was digested with appropriate restriction enzyme and subjected to agarose gel electrophoresis (low melting agarose 0.4%) at constant voltage of 6 V/cm. A slice of agarose gel containing the band of interest was cut out. Then DNAs were eluted from the slice by following procedure: the slice was melted at 65°C and added with an equal volume of water saturated phenol. After shaking, the mixture was centrifuged at 8,000 rpm at 4°C for 20 min. The same phenol extraction was repeated again. Then DNA was precipitated with 70% EtOH. The recovery of DNA from agarose was about 70%. Ligation of DNA was carried out with T4 DNA ligase at 12°C. ZEN2 (carrying both amber and opal suppressors) strain was transfected with the ligated DNAs.

The plaques were picked up by Pasteur pipettes and were put into a log-phase culture of Q1sup71. After complete lysis occurred, the phages were plated on appropriate bacterial host.

Determination of nucleotide sequence:

After cleaving T7 *up2* DNA by *KpnI* and *AvaI* restriction endonucleases, the *KpnI*-*AvaI* fragment (nucleotide 9,193-10,512) was separated by polyacrylamide gel electrophoresis and isolated from the gel and the 5' ends of fragments were labeled with

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[³²P] by using T4 polynucleotide kinase. The *AluI* fragment (nucleotide 9,802-10,199) or *HinfI* fragment (nucleotide 9,675-9,821) was isolated by the same procedure as *KpnI*-*AvaI* fragment and the 5' ends of their fragments were labeled with [³²P] similarly. After cleaved by *HhaI* (for the *AluI* fragment) or *TaqI* (for the *HinfI* fragment, the *AluI*-*HhaI* fragment (nucleotide 9,802-9,926) and *HinfI*-*TaqI* fragment (9,691-9,821) were isolated by polyacrylamide gel electrophoresis and sequenced by the method of Maxam and Gilbert (1979). The sequenced nucleotides were in r strand from *HinfI* site at nucleotide 9,824 to 9,769 and in l strand from *AluI* site, 9,802 to 9,861. The number of nucleotide and the nomenclature of T7 DNA strands are defined according to Dunn and Studier (1981).

Measurement of burst size:

The burst sizes of *opl* and newly isolated phages on several hosts were measured by the method described in Studier (1969).

Results

Isolation of a temperature sensitive mutant in the gene 2.5:

The mutant of the gene 2.5 of T7 phage was primarily isolated by Araki and Ogawa (1981a). This mutant, *up2*, made a truncated ssb protein which lacked 17 amino acids residues from C-terminal. (Araki, Shimizu and Ogawa 1990). This mutant protein could bind to double stranded DNA effectively as well as single-stranded DNA unlike the wild type ssb protein. In order to clear the roles of ssb protein in DNA replication, recombination or phage growth, I attempted to isolate a ssb mutant which lost the function completely by introducing an amber mutation or temperature sensitive mutation.

One temperature sensitive mutant (*ts2.5*) which could not complement *am1-am19* mutations at 42°C was isolated after screening 3000 mutagenized phages. The mutated gene was mapped by genetic cross (data not shown). The mutation site was between the gene 2 and the gene 3, and was determined by nucleotide sequencing. The mutant T7 phage DNA was digested with *BclI* and *BglII*, and 3.2 kb fragment which included T7 gene 2.5. was isolated after gel electrophoresis. The isolated fragment was inserted into a *BamHI* site on M13mp18 vector DNA (Figure 1.).

The nucleotide sequence of the cloned fragment on the M13mp18bb was determined by M13 methods. In this cloning, I always got the gene 2.5 on the vector in an opposite polarity but not in order polarity at the down stream of *lacZ* promoter. This region also could not be cloned into pMB9 (Campbell *et al.* 1978), pBR322 or pUC118 (our results). Moreover M13mp18bb is very unstable and about 80% of M13mp18bb had a deletion during overnight propagation. T7 *ts2.5* was found to have two mutations. One was the change of G to A of nucleotide 9,587, which caused the change, glycine to serine, and the other was the change of G to A of nucleotide 9,804, which changed trp codon (TGG) to the stop codon (ATG). The later mutation happened to affect the same amino acid as *op1* mutation occurred. The efficiency of plating (E.O.P.) of the mutant strains at 32°C or 42°C was shown in Table 3. E.O.P. of this mutant on ssb⁺ bacteria

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which has no amber suppression at 42°C was 10^{-5} , and E.O.P. on $ssb^+ su^+2$ was 10^{-3} . The result means that *ts2.5* mutant could not grow at 42°C if even host strain has the wild type *ssb* gene. Isolation of amber mutants in the gene 2.5 by *in vitro* mutagenesis: Two amber mutants in the gene 2.5 were isolated by *in vitro* mutagenesis. The outline of the isolation procedure is shown in Figure 2. The mutation sites of *am2.5-1* which is at the 15th amino acid from N terminal and *am2.5-2* which is at the 202nd amino acids from N terminal were confirmed by digestion with restriction enzymes, *Eco81I* (for *am2.5-1*) and *EcoT14I* (for *am2.5-2*). Both mutation sites had been designed to create a sensitive site for *Eco81I* or *EcoT14I*. The mutation sites and the nucleotide sequence and amino acids sequence are shown in Figure 3, and the plating efficiency of each phage is shown in Table 3. The efficiencies of *ts2.5*, *am2.5-1* and *am2.5-2* on non permissive condition were about 10^{-5} , except *ts2.5* on $594su^+2$ at 42°C. It was 10^{-3} and meant that the *ts2.5* was partially suppressed by su^+2 amber suppressor.

A nonsense mutation, *op1* in the *up2* mutant:

A T7 nonsense mutant which cannot grow on the su^+ssb defective strain but can grow on the su^+ssb^+ strain was isolated previously (Araki and Ogawa 1981). I tried to isolate a double mutant carrying both *up2* and other amber mutation such as *am3* or *am5*, but it was hard to get. On the other hand, T7 *J27a* strain (Studier 1981) which produced mutated *ssb* protein grows normally on *E.coli* $594ssb^-$ mutant strain. These results brought us to suspect that there were other mutations in *up2* strain. To remove the others mutation(s), genetic cross experiment were carried out between *up2* and *wild type* T7 phage. The crossed phages were selected on Q1 and 594. The progeny phage that can grow on Q1 but not on 594 was obtained by this experiment. This indicated that *up2* has other mutation(s) rather than gene 2.5. This phage which is dependent on suppressor but not on bacterial *ssb* was named as T7 *op1*.

Nucleotide sequencing of *op1* mutation:

The nucleotide sequence of *op1* mutation was determined. T7 *op1* makes a 10% smaller ssb protein in a molecular weight than wild type T7 ssb protein in a non-suppressive condition. Determination of amino acid composition of *op1* protein suggested that the mutation site of *op1* was near the carboxyl terminal region (in preparation of manuscript). Therefore, the nucleotide sequence around the c-terminal region was determined by Maxam and Gilbert method (1979). The determined nucleotide sequence were between *HinfI* site at nucleotide 9,824 and nucleotide 9,769 in r-strand and between *AluI* site at nucleotide 9,824 and nucleotide 9,861 in l-strand (Figure 4, see Materials and Methods). The result showed that *op1* mutation is change from TGG to TGA on l strand at nucleotide 9,805, and an opal mutation.

Mapping of outside mutations of ssb gene:

When genetic cross experiments between the *up2* mutant and other amber mutants, were carried out, various types of plaques (from very small plaques to normal plaques) were observed.

Moreover isolation of a double mutant, *up2* and an amber mutant in gene 5 was not successful by a genetic cross. Unidentified mutations other than *op1* mutation in the *up2* mutant were mapped by *in vitro* gene manipulation. As the other mutations may have no phenotype themselves, there is no way to identify the mutations by genetic cross.

Therefore, I tried to map them by substituting a various regions of the mutant genome with the corresponding regions of wild type phage *in vitro*. The substituted regions in the *up2* mutant phage DNA were shown in Figure 5. The solid bar shows original *up2* DNA and open bar shows a substituted region by the *wild type* DNA. The ZEN2 bacteria carrying an opal suppressor and an amber suppressor were transfected with the recombinant DNAs (AS1 to AS10). Obtained recombinant phages were propagated and the plating efficiency of each phage was examined. It was same for recombinant phages AS1, AS2 and AS3 on 594, and was one tenth of that of *up2* (Figure 5.). AS8 shows slightly reduced efficiency on 594 (0.5 of the frequency with *up2*). On the other hand, the efficiencies of AS4, AS5 and AS10 (*op1*) were 10^{-5} to that of *up2*.

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These results indicated that *up2* had probably two other mutations: one mutation (designated *S⁻¹*) lies between *Bgl*I-1 site and *Bst*II site (nucleotide 13,517 to 20,065), and the other (designated *S⁻²*) lies between *Bgl*I-2 and right end (nucleotide 35,931 to 39,936). These two regions corresponded to the map between gene 4 to gene 7 (*S⁻¹*) and lies between gene 17 to gene 20 (*S⁻²*). The plating efficiencies of AS7, which has both *S⁻¹* and *S⁻²* mutations, but not *op1* mutation, and AS9, which has *S⁻²* mutation alone, were same as that of wild type phage. It means that *S⁻¹* and *S⁻²* mutations themselves had no effect on plaque formation. To map two mutations more exactly, their linkage relationship with various genes were examined. T7 *op1S⁻¹ am9* was constructed and crossed with double mutants, *am1.am5*, *am1.am6* and *am1.am8* respectively, and the progeny phages were plated on an opal suppressor strain, CAJ64. Two hundred *am⁺* progeny of each cross were picked up and were stabbed onto the plates seeded by CAJ64 or 594. In the cross with *am1.am5*, *am1.am6* and *am1.am8*, 58%, 85% and 69% of progeny phages were able to grow on 594 respectively (Figure 6). If the *S⁻¹* mutation locates at the left of the gene 5 or at the right of the gene 8, the frequency of the recombinant phage which can grow on 594 should decrease or increase, in order, *am1.am5*, *am1.am6*, and *am1.am8*, respectively.

The observed frequency, however was the highest in the cross with *am1.am6*. This result indicated that *S⁻¹* was linked to the gene *am6* mutation. Next phage *op1S⁻¹.am6* phage was isolated by the cross of AS3 phage and *am6* phage. This recombinant phage cannot grow on 594, but its plating efficiency on 594^{su⁺2} is the same as that of *op1S⁻¹* on 594. If *S⁻¹* mutation was in gene 6, *op1.S⁻¹.am6* should have a double mutation in the gene 6. Therefore, *op1S⁻¹.am6* could not make suppressible protein of the gene 6 for *op1* mutation in 594. To test this possibility T7 *op1* and *op1S⁻¹.am6* were simultaneously spotted onto 594 loan. The complementation did not observed between them. However, when a complementation experiment was carried out using *op1S⁻¹.am5* or *op1S⁻¹.am8* instead of *op1S⁻¹.am6*, it was successfully observed (Table 4). These results indicated that *S⁻¹* was located in gene 6. By the similar procedure to the above, I ascertained that *S⁻²* was located in gene 18 (Table 4).

Plating efficiency and the burst size of *op1* mutant with or without the suppressor mutation:

The plating efficiency of *op1* mutant with or without the *S*⁻¹ or *S*⁻² mutation is shown in Table 5. For *op1* phage as well as 594*ssb-1* and 594*ssb-113*, it was 10⁻⁵ on 594 as compared with that on CAJ64. On the other hand, on FTP1327 which inserts Gly at the *opal* mutation site, the plating efficiency of *op1* was 0.8, but on 594su⁺²*op* which inserts Gln at the *opal* mutation site, it was 10⁻⁴. These results showed that defect of *op1* mutation was not substituted with the host *ssb* gene. The effect of *S*⁻¹ and *S*⁻² to the *op1* mutation was additive. Plating efficiency of *op1S*⁻¹ (*up2* without *S*⁻¹ suppressor) was 0.1, and that of *op1S*⁻² (*up2* without *S*⁻²) was 0.5. Interestingly, phages showed also a cold sensitive character. This is same for T7 *up2* phage. On *ssb* deficient bacterial strains (*ssb-1* and *ssb-113*), *up2*, *op1S*⁻¹ and *op1S*⁻² had the same very low plating efficiency. It suggested that the suppressor ability of the *S*⁻¹ and *S*⁻² mutation to the *op1* is dependent on the bacterial *ssb* function. In other word, T7 *wild type* phage does not use bacterial *ssb* in its growth cycle, and by bearing two suppressor mutations, T7 phage turns to be able to utilize bacterial *ssb* as well as own *ssb*.

Sedimentation analysis of intracellular T7 DNA:

Since the gene 6 product and gene 18 product are concerned with the formation and breakage of concatemer (Miller and Marion 1976), the effects of *S*⁻¹ and *S*⁻² to concatemer formation were examined. Concatemer formation was analyzed by sucrose gradient centrifugation. The results were shown in Figure 7. In *wild type* infected cells, almost all [³H]-labeled intracellular DNA cosedimented with [³²P]-labeled unit length T7 DNA added as a reference (Figure 7-(a)), and in *op1S*⁻¹ or *up2* infected cells, [³H]-labeled intracellular DNA sedimented broadly but their peaks were coincident with marker DNA (Figure 7-(b),(c)). On the other hand, in *op1S*⁻², several peaks were observed and sedimented far more faster than the reference DNA (Figure 7-(d)). This means that in the T7 *op1S*⁻² phage infected cells newly synthesized T7 DNA were hard

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to process to the unit length. It suggested that the combination of the mutated gene 6 exonuclease (*S⁻¹*) and mutated ssb (*opl*) recovered the ability of processing of the concatemer DNA.

The effect of *S⁻¹* and *S⁻²* to *ts2.5* and *am2.5-2*:

T7 *opl* was able to grow on *su⁻* strain, 594 if it carried additionally two other mutations, *S⁻¹* in gene 6 and *S⁻²* in gene 18. I examined the effect of those suppressors. *S⁻¹* and *S⁻²* were introduced to T7 *ts2.5* and *am2.5-2* by *in vitro* gene manipulation. Neither *ts2.5S^{-1S⁻²}* nor *am2.5-2S^{-1S⁻²}* mutant was able to grow on 594 at 42°C, or at 37°C (Table 3.) These results suggested that bacterial ssb cannot substitute for other T7 ssb mutations than *opl* even if the mutations *S⁻¹* and *S⁻²* were present.

Mutant ssb proteins in gel electrophoresis:

The mutant ssb proteins, of *opl* and *ts2.5* were analyzed by SDS gel electrophoresis. T7 *opl* and *ts2.5* mutant phages made a smaller ssb polypeptide than wild type as expected (Figure 8.).

T7 *opl* has an opal mutation, and in non-suppressive host it was expected to make smaller peptide with molecular weight 24,000 than wild type peptide, 25,562.

Although *ts2.5* peptide is expected to migrate as same as *opl* peptide in size, it migrated slower than *opl* peptide. The change to Ser at Gly144 in *ts2.5* peptide probably affects the mobility in the gel electrophoresis.

DNA synthesizing ability of the T7 ssb mutants:

The incorporation of [³H]-thymidine to the newly synthesized DNA in T7 ssb mutant infected cells were examined under no permissive condition. The results were shown in Figure 9. At 32°C and 37°C, *opl*, *J27a*, which was a gene 2.5 mutant isolated by Studier (1981) and *ts2.5* infected cells incorporated [³H]-Tdr less than wild type phage infected cells (Figure 9 (a),(b)). Incorporation of [³H]-Tdr in *opl* infected cells was recovered

by the presence of bacterial opal suppressor (Figure 9 (d)). *J27a* phage did not synthesize DNA at any temperature (Figure 9 (a)-(c)), and on any bacterial strain carrying suppressors (amber and opal) (Figure 9 (d)).

Both *am2.5-1* and *am2.5-2* phages could not synthesize DNA in a suppressor free strain, WD00, but synthesize DNA about a half amount of DNA synthesized in wild type phage infected cells (Figure 9 (e)). On the other hand, at 42°C *ts2.5* infected cells incorporated [³H]-Tdr as well as wild type infected cells after 10 min time lag (Figure 9 (c)). This DNA synthesis in *ts2.5* at 42°C is probably abnormal or uncontrolled. The reason is as follows: generally the synthesized DNAs of T7 phage in the phage infected cells were several times longer than unit length (concatemeric DNA). These concatemeric DNAs were processed by maturation proteins coded by the gene 18 and the gene 19. If T7 single-stranded DNA-binding protein is involved in this step, the concatemeric DNA was accumulated in *ssb* defective strain. The results of sedimentation analysis of newly synthesized DNA supported the above hypothesis (Figure 10). The concatemeric DNAs in the T7 *ts2.5* infected cells were not processed and accumulated at 42°C.

UV-sensitivity of mutant phages:

UV-sensitivity of *ts2.5* and *J27a* were measured. Phage *ts2.5* was irradiated by UV light at various doses and plated with indicator bacteria after appropriate dilution. The plates were incubated at 32°C for 30 min and some plates were transferred to 42°C and remaining plates were kept at 32°C as control. For phage *J27a*, the plates were incubated at 37°C. The results were shown in Figure 11. Phage *ts2.5* was 1.5 times as sensitive as wild type at 42°C, whereas at 32°C both have equal sensitivity to UV light (Figure 11 (a), (b)). *J27a* was also 1.5 times as sensitive as wild type to UV irradiation (Figure 11 (c)).

Recombination ability of T7 *ssb* mutants:

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Recombination frequency between gene 6 and gene 19 was measured using *ssb* mutant phages, *ts2.5*, *am2.5-1* and *am2.5-2* (Table 6.). The mutant *ts2.5* could carry out recombination at 32°C as well as wild type (about 25%). At 42°C, however it reduced the frequency to about 1/3 of that of *wild type*. Both *am2.5-1* mutant and *am2.5-2* mutant also reduced the frequency similarly under nonpermissive condition. These results showed that *ssb* protein participated in genetic recombination. In the case of the *op1* mutant, the burst size under the nonpermissive condition was very low, and the high recombination frequency obtained might result from revertant phages.

Formation of intermediate DNA molecules in recombination:

The recombination intermediate DNA molecules were isolated by CsCl density gradient centrifugation (Tsuji moto and Ogawa 1977). Bacteria, 594 were infected with [³²P]-labeled T7 *ts2.5* phage and [³H]BU-labeled T7 *ts2.5* phage at 32°C or 42°C. The phage infected cells were incubated for 10 min, and DNA molecules were isolated after 3 cycles of freezing and thawing. Then isolated DNA molecules were centrifuged in CsCl density gradient. After the first centrifugation, about 1.5 % and 1.3 % of input [³²P]-radioactivity were recovered around half-heavy position at 32°C and 42°C, respectively (data not shown). In the second centrifugation, however about 55 % of radioactivity was recovered at half-heavy position at 32°C, whereas few radioactivity was found at half-heavy position at 42°C (Figure 12). These results showed that the intermediates of recombination were not formed in the *ts2.5* infected cells at 42°C.

Discussion

In this paper, three mutants of gene 2.5 of T7 phage were isolated: one was, a temperature sensitive mutant, T7 *ts2.5*, obtained after NTG treatment to the T7 infected cells; and other two were amber mutants, T7 *am2.5-1* and *am2.5-2*, which were obtained by *in vitro* mutagenesis.

All these mutants could not make viable progeny phage under the restrictive condition, at 42°C or in suppressor free host bacteria. This means that T7 phage ssb protein is essential for phage growth, and that bacterial ssb protein cannot replace T7 phage ssb. These results prompted us to analyze T7 *up2* mutant whose growth was dependent on host ssb. T7 *up2* was found to have an opal mutation *op1* not but an amber mutation as speculated previously (Araki and Ogawa 1981) in the gene 2.5 by nucleotide sequencing of the mutation. Furthermore, it had additional two suppressor mutations other than the opal mutation in the gene 2.5. One suppressor mutation was in the gene 6; the other was in the gene 18. T7 *op1* strain which had the opal mutation alone in the gene 2.5 could grow on several host strains such as Q1supE (Signer *et al*) a derivative from C600 (Appleyard, 1954) and W3110 which were believed not to carry an opal suppressor. The analysis of the protein synthesized in the mutant phages infected cells showed that *op1* phage made an intact size peptide in Q1 as well as in CAJ64 a standard opal suppressor strain, but in 594 a smaller size peptide than wild type. as expected from the mutation site (Araki, Shimizu and Ogawa in preparation of manuscript).

Since the amber suppressor cannot suppress an opal mutation in general, the suppression of *op1* mutation by the supE stain was seemed to be resulted from the intrinsic ambiguity of tRNA^{trp} not by the amber suppression. Actually the efficiency of plating of *op1* strain Q1 was low and about 0.1 to that on CAJ64. The burst sizes of *op1* strain in these bacterial strains were 5 and 130 respectively. The suppressor ability of Q1 for the *op1* mutation seems to be as low as an amber mutant on a suppressor free

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strain, since the burst size of a rigid amber mutant *am6* in 594 was nearly one particle/cell.

Growth of T7 *up2* strain was dependent on the bacterial *ssb* gene function (Table 5). This suggested that bacterial *ssb* play a some role for growth of T7 *up2* strain. One explanation is that by the mutations of *S⁻¹* and *S⁻²*, T7 *opl* mutant acquired an ability to utilize able to use bacterial *ssb* for the growth. The *opl* mutant protein complexed with T7 *S⁻¹* and *S⁻²* may interact with host *ssb* to compensate for the defect of the T7 *ssb* protein. Above explanation was confirmed by the fact that the suppression by the mutations *S⁻¹* and *S⁻²* was specific for the mutation *opl* and two mutations could not restore the inviability of other T7 *ssb* mutants (*ts2.5* and *am2.5-2*) under the nonpermissive condition.

This suggests that suppressors probably interact with T7 *ssb*.

Since the suppressors were the mutations in the gene 6 and gene 18, which participated in the formation and cleavage of concatemers respectively (Miller and Marion 1976; Richardson *et al* 1987), T7 *ssb* might be involved in the maturation of T7 DNA by interacting with these genes products.

The *ssb* protein synthesized by the infection of *J27a* phage migrated faster than the wild type protein in SDS gel electrophoresis but any bacterial nonsense suppressors could not affect the migration of *J27a* *ssb* protein (Studier, 1981), and *J27a* *ssb* protein acted *in vitro* in a manner similar to the *wild type* protein. *J27a* phage may have a missence mutation in the *ssb* gene not but a deletion mutation as speculated (Studier, 1981) and make a full length protein with single amino acids change, since the change of single amino acid in T7 *ssb* protein could affect the mobility in the gel electrophoresis greatly (Figure 9).

T7 *ts2.5* was found to have two mutations: one was a missence mutation, Gly144 to serine; another was a nonsense amber mutation at Trp216. T7 *am2.5-1* and *am2.5-2* would make 15 amino acids and 200 amino acids peptides, respectively.

The correlation between DNA synthesis (Figure 9-(e)) and progeny formation (Table 3.) by infection of both amber mutants (*am2.5-1* and *am2.5-2*) was reasonable.

However, a large amount of DNA synthesis in T7 *ts2.5*-infected cells at 42°C (Figure 9-(c)) was unexpected, because T7 *ts2.5* could not make viable progeny at 42°C. This discrepancy may be caused by the induction of abnormal DNA synthesis in T7 *ts2.5*-infected cells at 42°C. In the T7 *ts2.5* infected cells, the concatemeric DNAs were accumulated (Figure 10.). This accumulation of the concatemeric DNAs was explained by two ways.

One explanation is that in the late period of T7 wild type phage infection, newly synthesized concatemeric DNAs are processed by the late gene products. In T7 *ts2.5* infected cells, however, their processing does not operate at 42°C. The other explanation is that the mode of DNA synthesis in T7 *ts2.5* infected cells is different from that in *wild type* infected cells, and the abnormal molecules such as branched DNA which can not be processed by the T7 late genes are accumulated in *ts2.5* infected cells. At present, the former is more likely, because T7 our results suggested that T7 *ssb* could interacts with the gene *I8* product that is concerned with the processing of concatemeric DNA. The gene *I8* products probably require T7 *ssb* function to process concatemeric DNA molecules.

In conclusion, T7 *ssb* protein is essential for phage growth and it takes parts in several reactions of DNA metabolism such as replication, recombination and repair.

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Legends

Table 3.

The efficiency of plating.

The value was relative to the plaques on 594 of T7 wild type at 37°C.

-: not test *: small plaque

Table 4.

Complementation Test

The phage listed in row and the phage listed in column were spotted on 594 loan simultaneously to test the complementation between them.

+: complemented -: not complemented

Table 5.

Efficiency of plating

Table 6.

Recombination frequency

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Figure 3.

Mutation sites and diagram of gene 2.5 mutants

- (a) mutation sites of gene 2.5 mutants
- (b) diagram of mutants

Figure 4.

Mutation site of T7 *op1* strain in the gene 2.5

DNA fragments containing nucleotides 9,691-9,821 were isolated by polyacrylamide gel electrophoresis and sequenced by the method of Maxam and Gilbert (1979). The nucleotides sequenced were in r-strand from *HinfI* site at nucleotide 9,824 to 9,769 and in l-strand from *AluI* site at nucleotide 9,802 to 9,861. The number of nucleotide and the nomenclature of T7 DNA strands are defined according to Dunn and Studier (1981).

Figure 5.

Mapping of suppressors in T7 *up2* by a partial substitution with the wild type strain

Bold lines show the DNA from T7 *up2* and open lines show the DNA from T7 wild type. The value of light column of the figure was the relative E.O.P.s of the recombinant phages (AS1 to AS10) to *up2* phage at 37°C and was calculated by the following equation:

$$V(n) = \frac{(\text{plaques of ASn on 594})/(\text{plaques of ASn on CAJ64})}{(\text{plaques of up2 on 594})/(\text{plaques of up2 on CAJ64})}$$

n=1 to 10

Figure 6.

Linkage between suppressor mutation S-1 and amber mutations

T7 *opIS-1 am9* was constructed and crossed with double mutants, *aml.am5*, *aml.am6* and *aml.am8* respectively, and the progeny phages were plated on an opal suppressor strain, CAJ64. Two hundred *am*⁺ progeny of each cross were picked up and were stabbed onto the plates seeded by CAJ64 or 594. The value was the ratio of the plaques on 594 to the plaques on CAJ64.

Figure 7.

Analysis of intracellular DNAs in mutants T7 infected cells

E.coli 594 was grown at 30°C to 3×10^8 cells/ml in M9 medium. The Cells were infected with indicated phages at a multiplicity of infection of 10. At 8 min after infection [³H]-thymidine (1.67 GBq/μmol) was added to each infected culture. At 10 min after infection samples were ice chilled with TEN-buffer. The DNA was extracted and analyzed by neutral sucrose gradient sedimentation. After centrifugation the sample was collected from bottom of tubes and acid-insoluble radioactivity was determined. arrow indicates the sedimented position of monomer T7 DNA.

(a) wt (b) *up2* (c) *opIS-1* (d) *opIS-2*

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Figure 8.

Analysis of mutant proteins of *ssb*

Bacterial strain 594 was grown in M9 medium supplemented with 0.2% Glucose and 0.2% Casamino Acid (Difco) to the density of 3×10^8 cells/ml. The culture (5 ml) was irradiated by UV light and after culturing for 5 min, T7 phage was infected at a multiplicity of 20. After 15 min of infection, infected cells were collected by centrifugation at 8000 rpm at 4°C for 10 min. The pellet was suspended in 0.1 ml of SDS buffer. The sample was subjected to SDS gel electrophoresis by Leamli's method. The position of gene 2.5 product was deduced by the position of purified *ssb* protein which was electrophorized simultaneously.

lane (a):*wild type* lane (b):*ts2.5* lane (c):*am 6* lane (d):*op1*

The upper arrow shows the position of wild type *ssb* protein, which identified by purified *ssb* protein. The lower arrow shows the position of *op1* protein.

Figure 9.

Measurement of DNA synthesis

Bacterial strains WD00 or CAJ64thyA was grown to the density of 3×10^8 cells/ml in M9 medium supplemented with 0.2% Glucose, 0.2% Casamino Acids, 2 µg/ml thymine and 1 µCi/ml [³H]-thymidine Phages were infected to each culture at a multiplicity of 10.

At a time indicated, 100 µl-samples were removed and equal volume of 10% TCA was added to the samples and chilled on ice and the acid-insoluble radioactivity was determined.

- (a):(○) *wt* (●) *op1* (△) *J27a* (▲) *ts2.5* in WD00 at 32°C
- (b):(○) *wt* (●) *op1* (△) *J27a* (▲) *ts2.5* in WD00 at 37°C
- (c):(○) *wt* (●) *op1* (△) *J27a* (▲) *ts2.5* in WD00 at 42°C
- (d):(○) *wt* (●) *op1* (△) *J27a* (▲) *up2* in Q1sup71 at 37°C
- (e):(□) *wt* in WD00 (○) *am2.5-1* in WD003 (●) *am2.5-1* in WD00
(△) *am2.5-2* in WD001 (▲) *am2.5-2* in WD00

Figure 10.

Analysis of intracellular DNAs of T7 *ts2.5* infected cells

E.coli 594 was grown at 30°C to 3×10^8 cells/ml in M9 medium.

The Cells were infected with indicated phages at a multiplicity of infection of 10. At 8 min after infection [^3H]-thymidine (1.67GBq/ μmol) was added to each infected culture. At 10 min after infection samples were ice chilled with TEN-buffer. The DNA was extracted and analyzed by neutral sucrose gradient sedimentation After centrifugation the sample was collected from bottom of tubes and acid-insoluble radioactivity was determined. arrow indicates monomer T7 DNA.

(a): sedimentation pattern at 32°C

(b): sedimentation pattern at 42°C

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Figure 11.

UV sensitivity of ssb mutants

Phage stocks were diluted to 10^9 phages/ml in T7 buffer and irradiated in a 100 mm Petri dish with constant agitation under UVS 15-watt low-pressure germicidal lamp at dose rates of about $1.0 \text{ J m}^{-2} \text{ sec}^{-1}$. Irradiated samples plated were incubated under dark room to avoid photoreactivation.

(a):*ts2.5* at 32°C (b):*ts2.5* at 42°C (c):*J27a* at 32°C

Figure 12.

Recentrifugation profile of T7 DNA

Centrifugation was carried out by the procedure described in Materials and Methods.

(a): infected at 32°C (b): infected at 42°C

Tables

Table 1.

E.coli K12 strains

strain	relevant genotype	reference
594	<i>sup</i> ⁺	Campbell(1965)
594 <i>su</i> ⁺ 7	<i>psu</i> ⁺ 7	this work
Q1	<i>supE</i>	obtained from Dr.E. Signer
Q1 <i>sup</i> 71	<i>supE sup</i> 71	this work
C600	<i>supE thr leu</i>	Appleyard(1954)
C600 <i>thyA</i>	<i>supE thr leu thyA deo</i>	laboratory collection
C600 <i>r⁻m⁻recB</i>	<i>supE thr leu</i>	laboratory collection
ZEN2	<i>r-m- recB sup</i> 71 <i>supE</i>	this work
594 <i>ssb</i> -1	<i>sup</i> ⁺ <i>thyA deo</i>	H.Araki and H.Ogawa(1981)
594 <i>ssb</i> -113	<i>sup</i> ⁺ <i>thyA deo</i>	H.Araki and H.Ogawa(1981)
C600 <i>ssb</i> -1	<i>supE thyA deo</i>	H.Araki and H.Ogawa(1981)
C600 <i>ssb</i> -113	<i>supE thyA deo</i>	H.Araki and H.Ogawa(1981)
011'	<i>supE thyA deo gal str^r</i>	obtained from Dr.W.Studier
CAJ64	<i>sup</i> 71	obtained from Dr.H. Inokuchi
FTP1327	<i>glyT</i>	obtained from Dr.H. Inokuchi
594 <i>su</i> ⁺ 2 <i>op</i>	<i>psu</i> ⁺ 2UGA	obtained from Dr.H. Inokuchi
WD00	<i>thyA deo sup</i> ⁺	laboratory collection
WD001	<i>thyA deo su</i> ⁺ 1	laboratory collection
WD002	<i>thyA deo su</i> ⁺ 2	laboratory collection
WD003	<i>thyA deo su</i> ⁺ 3	laboratory collection

Table 2.

T7 phage strains used in this study

phage	gene	relevant defective characteristics
<i>up2</i>	2.5	single-stranded DNA-binding protein
<i>opl</i>	2.5	single-stranded DNA-binding protein
<i>am1-193</i>	1	RNA polymerase
<i>am2-64</i>	2	Inactivates host RNA polymerase
<i>am3-29</i>	3	endonuclease
<i>am4-208</i>	4	DNA primase
<i>am5-28</i>	5	DNA polymerase
<i>am6-233</i>	6	5'-exonuclease
<i>am8-11</i>	8	Head-tail protein
<i>am9-17</i>	9	Head assembly protein
<i>am12-3</i>	12	Tail protein
<i>am13-149</i>	13	Internal virion protein
<i>am14-140</i>	14	Internal virion protein
<i>am15-31</i>	15	Head protein
<i>am16-9</i>	16	Head protein
<i>am17-290</i>	17	Tail-fiber protein
<i>am18-182</i>	18	DNA maturation
<i>am19-10</i>	19	DNA maturation
<i>oplS-1</i>		this work
<i>oplS-2</i>		this work

Table 3.

Efficiency of Plating

host	CAJ (37°C 42°C)		594su+2 (37°C 42°C)		594	WD001 (37°C)	WD002	WD003
phage								
<i>Wild type</i>	1	1	0.9	0.9	0.9	1	0.9	1
<i>ts2.5</i>	1	10 ⁻⁵	1	10 ⁻³	10 ⁻⁵	10 ⁻⁴	-	-
<i>ts2.5S-1S-2</i>	1	10 ⁻⁵	1	10 ⁻³	10 ⁻⁵	-	-	-
<i>am2.5-1</i>	-	-	-	-	7x10 ⁻⁵	-	-	1
<i>am2.5-2</i>	-	-	-	-	2x10 ⁻⁵	1	-	-
<i>am2.5-2S-1S-2</i>	-	-	-	-	3x10 ⁻⁵	1	-	-
<i>op1</i>	1	1	10 ⁻⁴	10 ⁻⁴	10 ⁻⁴	-	-	-
<i>J27a</i>	1	1	0.9	0.8	0.9	-	-	-
<i>am6</i>	10 ⁻⁵	10 ⁻⁵	1	1	10 ⁻⁵	0.8	1	0.8

Table 4.

Complementation Test

A)

	<i>opl</i>	<i>am5</i>	<i>am16</i>
<i>oplS-1S+2am5</i>	+	-	+
<i>am15</i>	+	+	+

	<i>opl</i>	<i>am6</i>	<i>am16</i>
<i>oplS-1S+2am6</i>	-	-	+
<i>am15</i>	+	+	+

B)

	<i>opl</i>	<i>am17</i>	<i>am16</i>
<i>oplS+1S-2am17</i>	+	-	+
<i>am15</i>	+	+	+

	<i>opl</i>	<i>am18</i>	<i>am16</i>
<i>oplS+1S-2am18</i>	-	-	+
<i>am15</i>	+	+	+

Table 5.

Efficiency of Plating

host	CAJ (37°C)	Q1 ()	594	594 ()	594 <i>ssb-1</i> (32°C)	594 <i>ssb-113</i> ()
phage						
<i>Wild type</i>	1	1	1	0.7	1.6	1.5
<i>up2</i>	1	1	0.8	10 ⁻²	10 ⁻³	10 ⁻⁴
<i>opl</i>	1	0.5	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵	10 ⁻⁵
<i>opl.S-1</i>	1	-	0.1	5x10 ⁻³	10 ⁻³	10 ⁻⁴
<i>opl.S-2</i>	1	-	0.5	10 ⁻⁴	10 ⁻³	10 ⁻⁴
<i>am6</i>	10 ⁻⁶	1	10 ⁻⁶	10 ⁻⁶	10 ⁻⁶	10 ⁻⁶

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Table 6.

Recombination Frequency

	permissive		nonpermissive	
	rec. freq.	burst size	rec. freq.	burst size
	%		%	
<i>wild type</i> (<i>am6 x am19</i>)	27.5	120	18.6	60
<i>up2</i> (<i>up2 am6 x</i> <i>up2 am19</i>)	27.1	50	4.2	20
<i>opl</i> (<i>opl am6 x</i> <i>opl am19</i>)	22.5	60	14.1	0.2
<i>ts2.5</i> (<i>ts2.5 am6 x</i> <i>ts2.5 am19</i>)	29.0	45	5.3	2.1
<i>am2.5-1</i> (<i>am2.5-1 am6 x</i> <i>am2.5-1 am19</i>)	20.1	47	5.6	1.5
<i>am2.5-2</i> (<i>am2.5-2 am6 x</i> <i>am2.5-2 am19</i>)	22.0	60	7.0	1.6

Figures

Structure of M13mp18bb

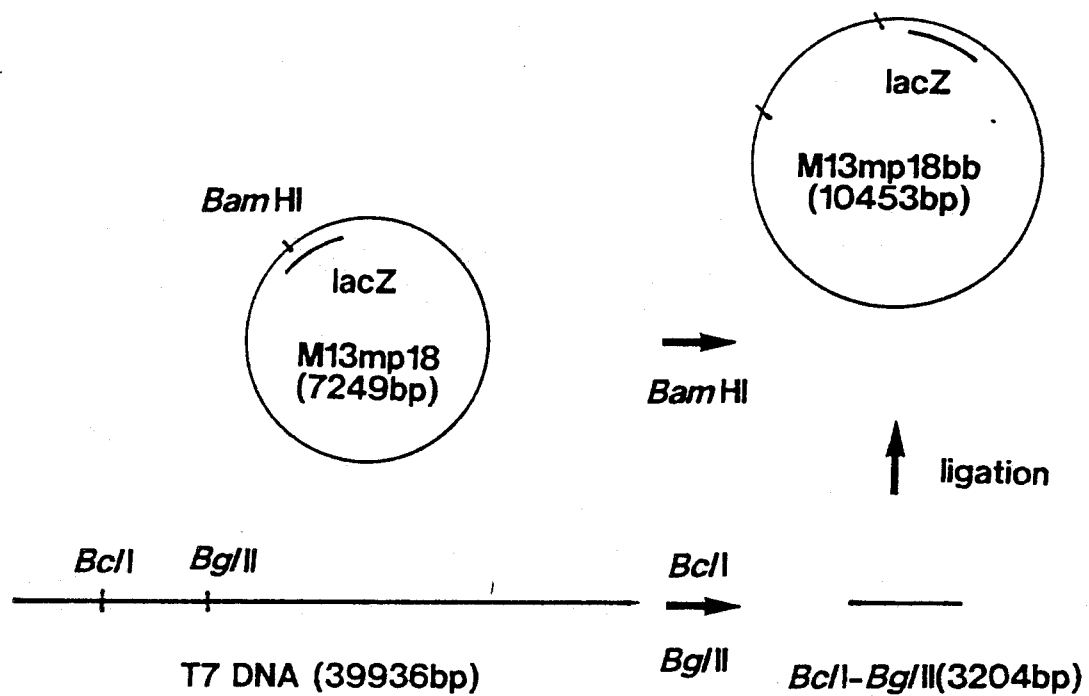


Figure 1.

Scheme for mutagenesis of gene 2.5

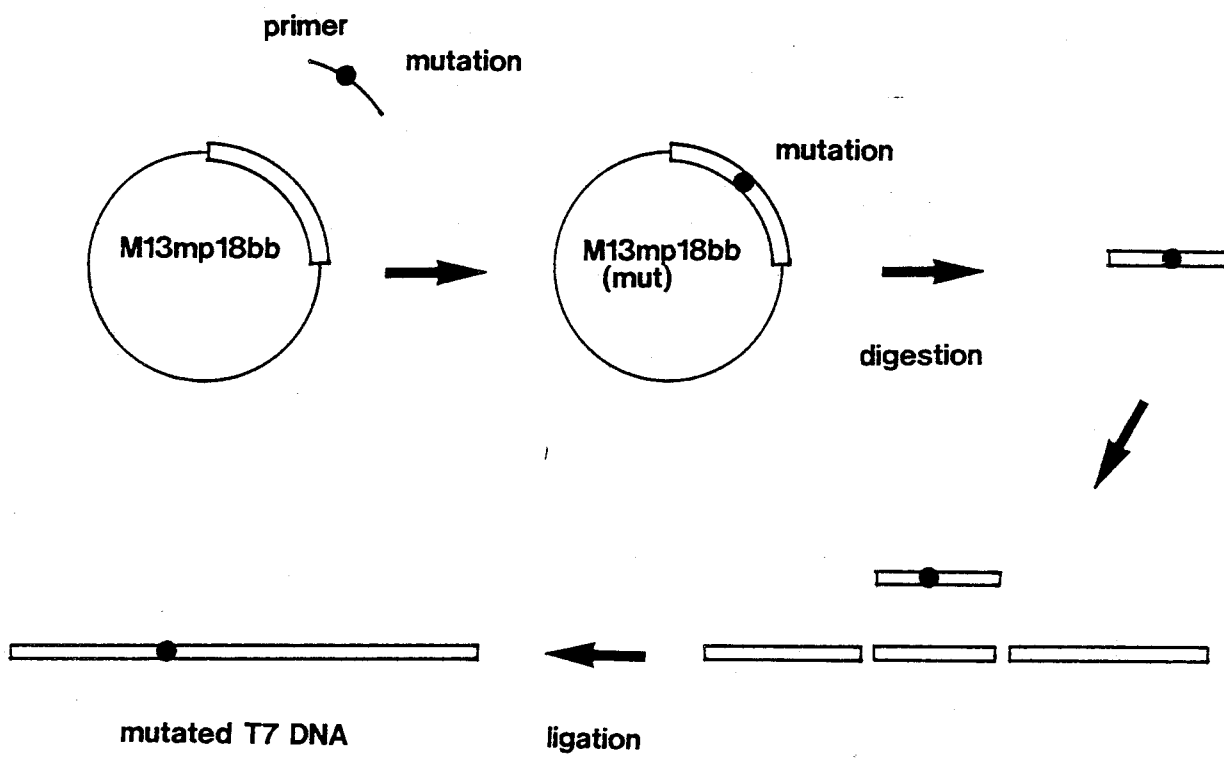


Figure 2.

Mutation sites and diagram of gene 2.5 mutants

(a) mutation sites of gene 2.5

(Eco81I)

1 10 G (Tyr-amber)

ATGGCTAAGAAGATTTTCACCTCTGCGCTGGGTACCGCTGAACCTTACGCTTACATCGCC

MetAlaLysLysIlePheThrSerAlaLeuGlyThrAlaGluProTyrAlaTyrIleAla

***am2.5-1

21 30 40

AAGCCGGACTACGGCAACGAAGAGCGTGGCTTTGGGAACCCTCGTGGTGTCTATAAAGTT

LysProAspTyrGlyAsnGluGluArgGlyPheGlyAsnProArgGlyValTyrLysVal

41 50 60

GACCTGACTATTCCCAACAAAGACCCGCGCTGCCAGCGTATGGTTCGATGAAATCGTGAAG

AspLeuThrIleProAsnLysAspProArgCysGlnArgMetValAspGluIleValLys

61 70 80

TGTCACGAAGAGGCTTATGCTGCTGCCGTTGAGGAATACGAAGCTAATCCACCTGCTGTA

CysTyrGluGluAlaTyrAlaAlaAlaValGluGluTyrGluAlaAsnProProAlaVal

81 90 100

GCTCGTGGTAAGAAACCGCTGAAACCGTATGAGGGTGACATGCCGTTCTTCGATAACGGT

AlaArgGlyLysLysProLeuLysProTyrGluGlyAspMetProPhePheAspAsnGly

101 110 120

GACGGTACGACTACCTTTAAGTTCAAATGCTACGCGTCTTTCCAAGACAAGAAGACCAAA

AspGlyThrThrThrPheLysPheLysCysTyrAlaSerPheGlnAspLysLysThrLys

121 130 140

GAGACCAAGCACATCAATCTGGTTGTGGTTGACTCAAAAAGGTAAGAAGATGGAAGACGTT

GluThrLysTyrIleAsnLeuValValValAspSerLysGlyLysLysMetGluAspVal

141 A (Gly-Ser) ts2.5 150 160

CCGATTATCGGTGGTGGCTCTAAGCTGAAAGTTAAATATTCTCTGGTTCCATACAAGTGG

ProIleIleGlyGlyGlySerLysLeuLysValLysTyrSerLeuValProTyrLysTrp

161 170 180

AACACTGCTGTAGGTGCGAGCGTTAAGCTGCAACTGGAATCCGTGATGCTGGTCAACTG

AsnThrAlaValGlyAlaSerValLysLeuGlnLeuGluSerValMetLeuValGluLeu

181 190 200

GCTACCTTTGGTGGCGGTGAAGACGATTGGGCTGACGAAGTTGAAGAGAACGGCTATGTT

AlaThrPheGlyGlyGlyGluAspAspTrpAlaAspGluValGluGluAsnGlyTyrVal

(EcoT14I)

201 AG (Ser-amber) 210 220

GCCTCTGGTTCTGCCAAAGCGAGCAAACCACGCGACGAAGAAAGCTGGGACGAAGACGAC

AlaSerGlySerAlaLysAlaSerLysProArgAspGluGluSerTrpAspGluAspAsp

***am2.5-2

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GAAGAGTCCGAGGAAGCAGACGAAGACGGAGACTTCTAA A (Trp-opal) opl

GluGluSerGluGluAlaAspGluAspGlyAspPhe*** A (Trp-amber) ts2.5

Figure 3.

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(b) diagram of mutants

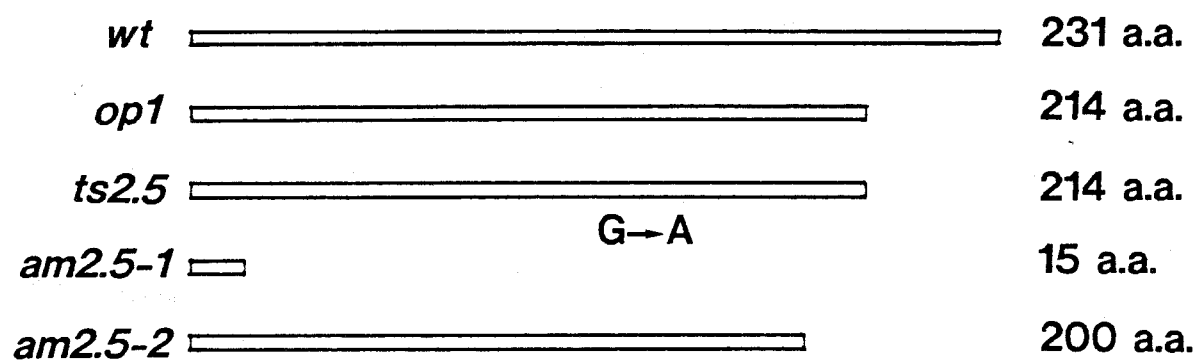


Figure 3 (cont.).

Mutation site of T7 *opl* strain of gene 2.5

10	20	30	40	50	60
ATGGCTAAGA	AGATTTTCAC	CTCTGCGCTG	GGTACCGCTG	AACCTTACGC	TTACATCGCC

70	80	90	100	110	120
AAGCCGGACT	ACGGCAACGA	AGAGCGTGGC	TTTGGAAC	CTCGTGGTGT	CTATAAAGTT
130	140	150	160	170	180
GACCTGACTA	TTCCCAACAA	AGACCCGCGC	TGCCAGCGTA	TGGTCGATGA	AATCGTGAAG
190	200	210	220	230	240
TGTCACGAAG	AGGCTTATGC	TGCTGCCGTT	GAGGAATACG	AAGCTAATCC	ACCTGCTGTA
250	260	270	280	290	300
GCTCGTGGTA	AGAAACCGCT	GAAACCGTAT	GAGGGTGACA	TGCCGTTCTT	CGATAACGGT
310	320	330	340	350	360
GACGGTACGA	CTACCTTTAA	GTTCAAATGC	TACGCGTCTT	TCCAAGACAA	GAAGACCAAA
370	380	390	400	410	420
GAGACCAAGC	ACATCAATCT	GGTTGTGGTT	GA CTCAAAG	GTAAGAAGAT	GGAAGACGTT
430	440	450	460	470	480
CCGATTATCG	GTGGTGGCTC	TAAGCTGAAA	GTTAAATATT	CTCTGGTTCC	ATACAAGTGG
490	500	510	520	530	540
AACACTGCTG	TAGGTGCGAG	CGTTAAGCTG	CAACTGGAAT	CCGTGATGCT	GGTCGAAGTG
					TaqI
550	560	570	580	590	600
GCTACCTTTG	GTGGCGGTGA	AGACGATTGG	GCTGACGAAG	TTGAAGAGAA	CGGCTATGTT
610	620	630	640	650	660
GCCTCTGGTT	CTGCCAAAGC	GAGCAAACCA	CGCGACGAAG	AAAGCTGGGA	CGAAGACGAC
				AluI	
670	680	690	699	A (opal, opl)	
GAAGAGTCCG	AGGAAGCAGA	CGAAGACGGA	GACTTCTAA		
HinfII			...		

Figure 4.

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Mapping of suppressors in T7 *up2* by a partial substitution with the *wild type* strain

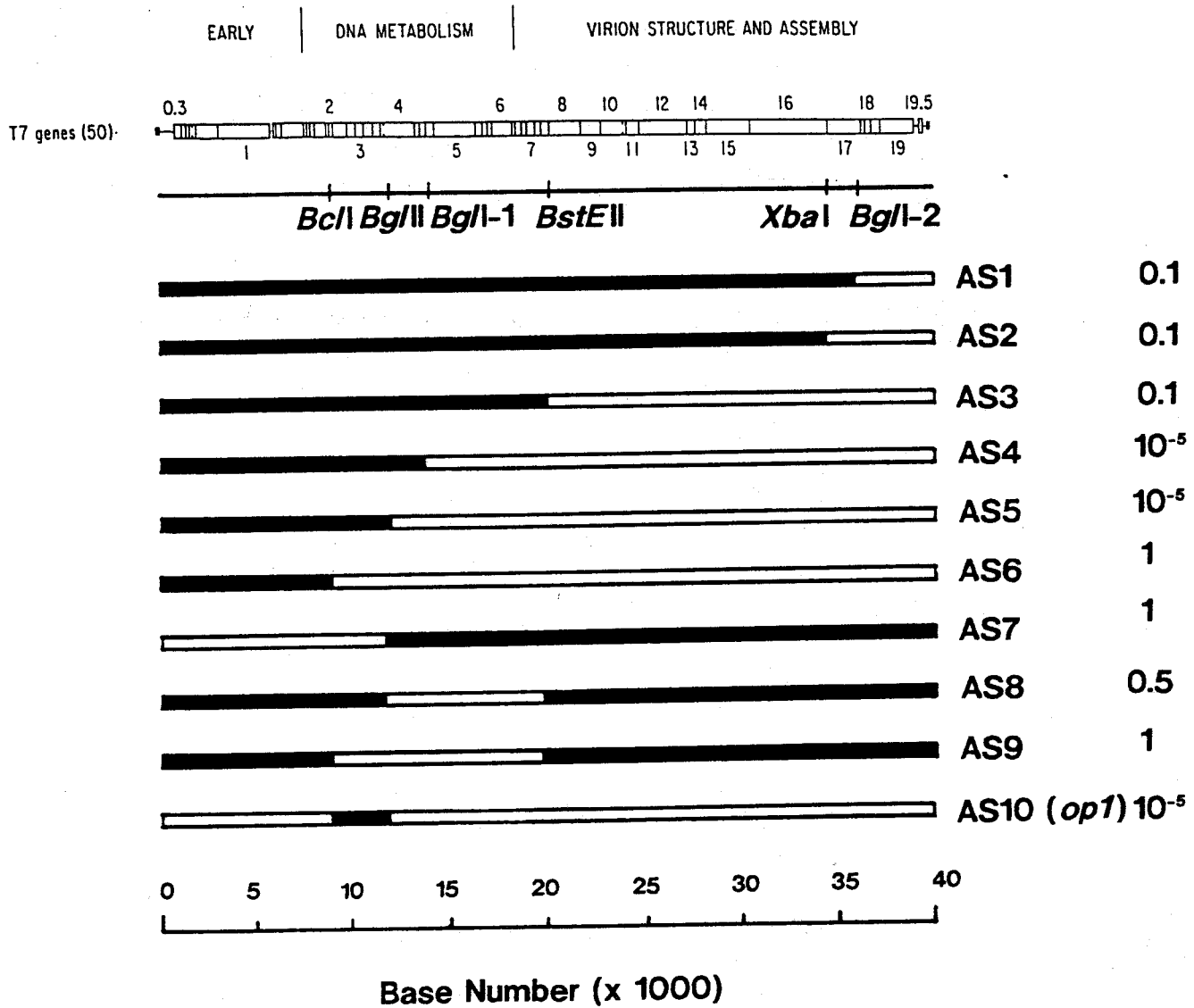
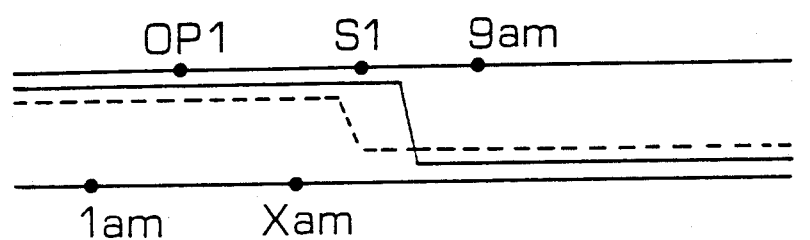


Figure 5.

Linkage between suppressor mutation *S-1* and amber mutations

Marker	594/CAJ64
<i>am5</i>	0.58
<i>am6</i>	0.95
<i>am8</i>	0.68

Figure 6.

Analysis of intracellular DNAs in T7 mutants infected cells

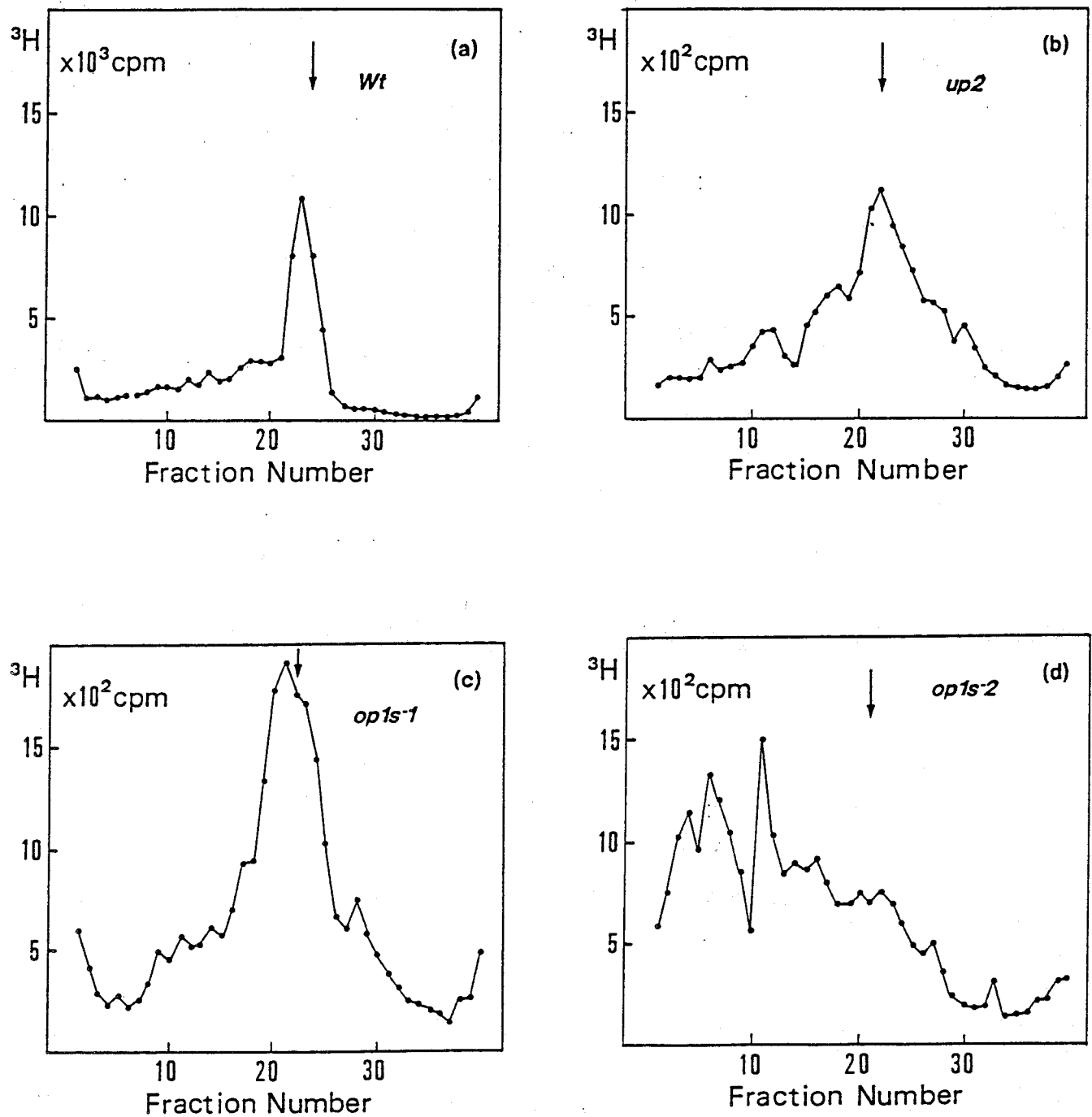


Figure 7.

Analysis of mutant proteins of ssb

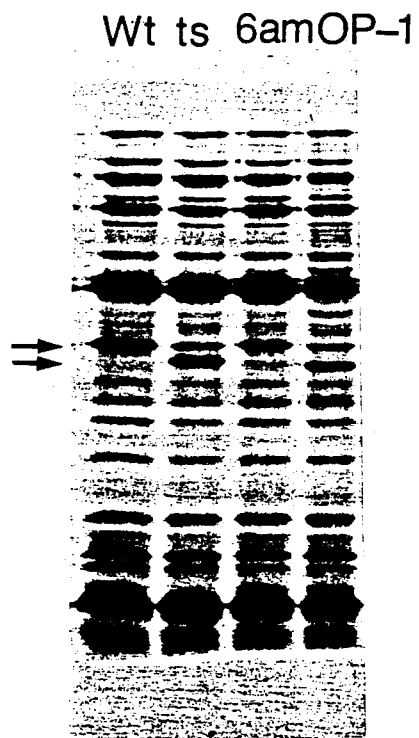


Figure 8.

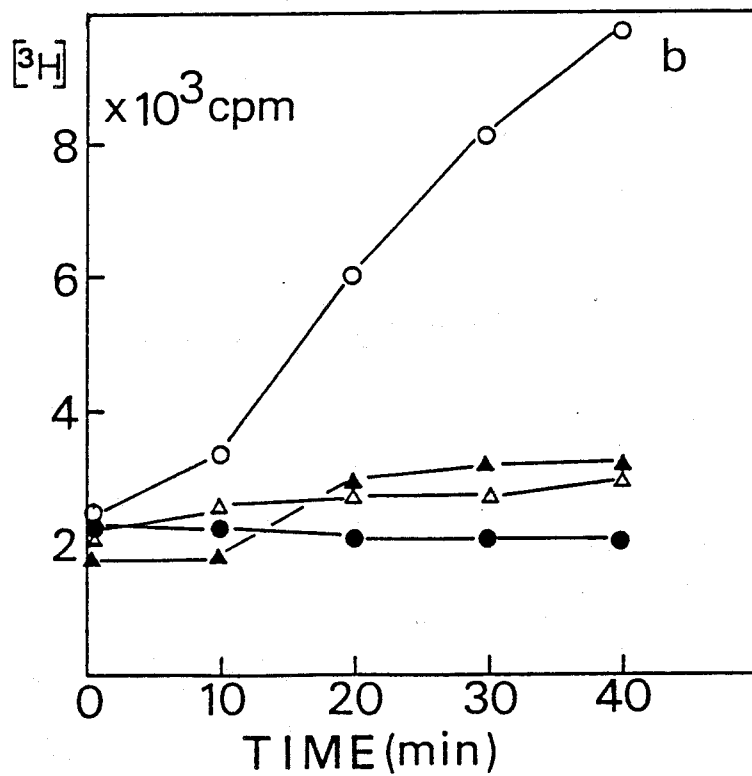
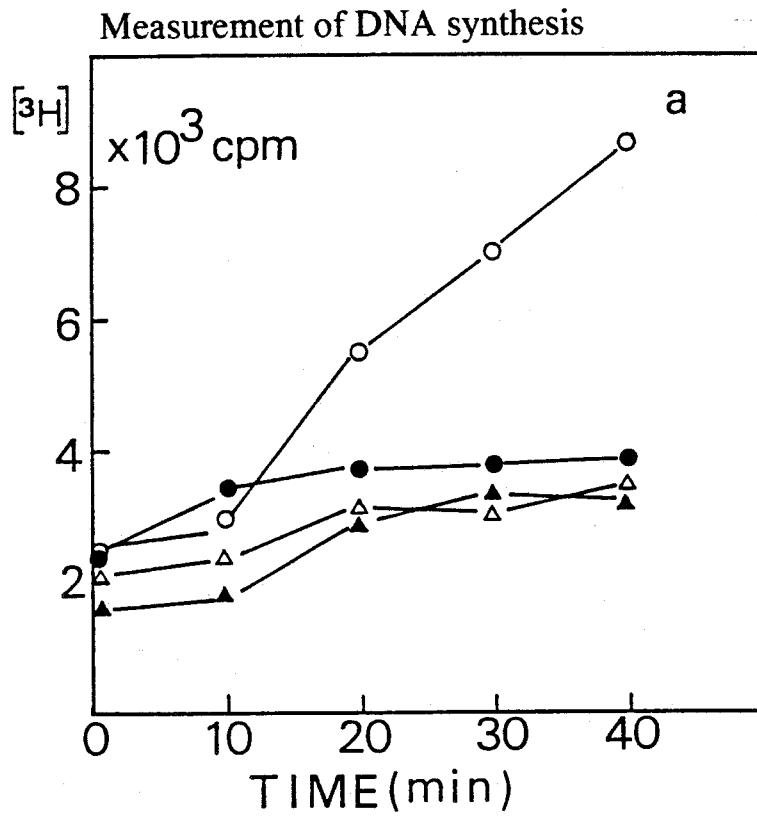


Figure 9.

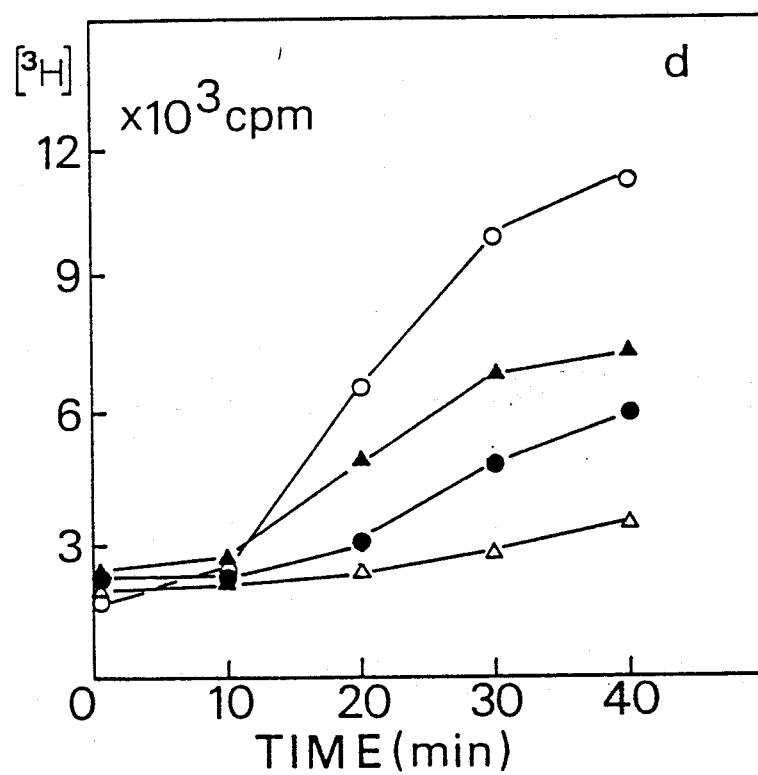
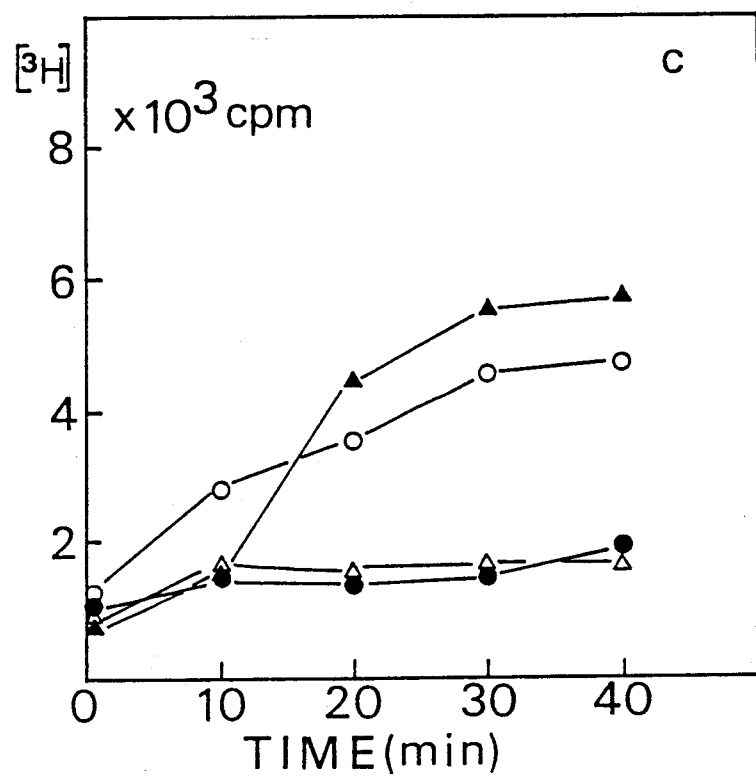


Figure 9. (cont.)

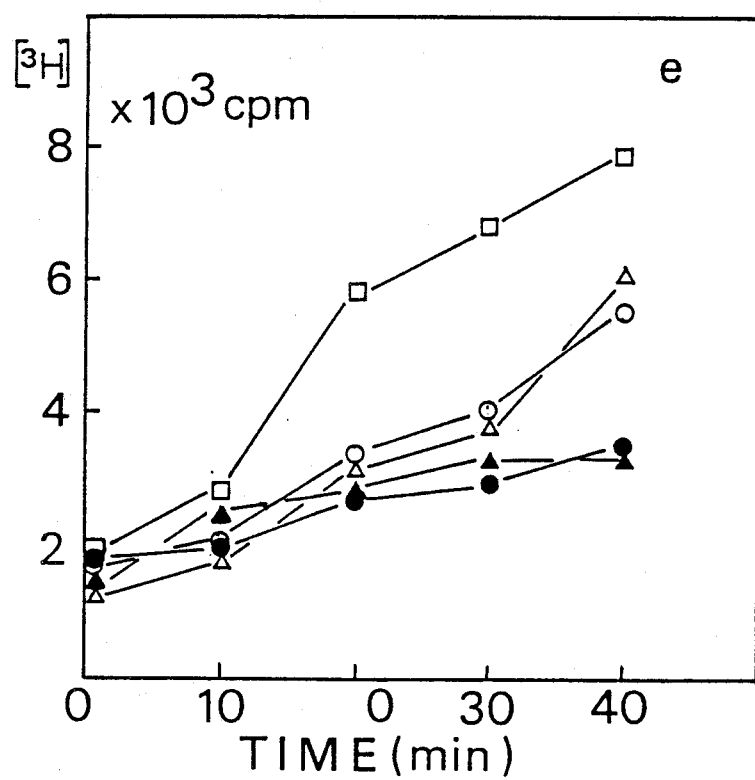


Figure 9. (cont.)

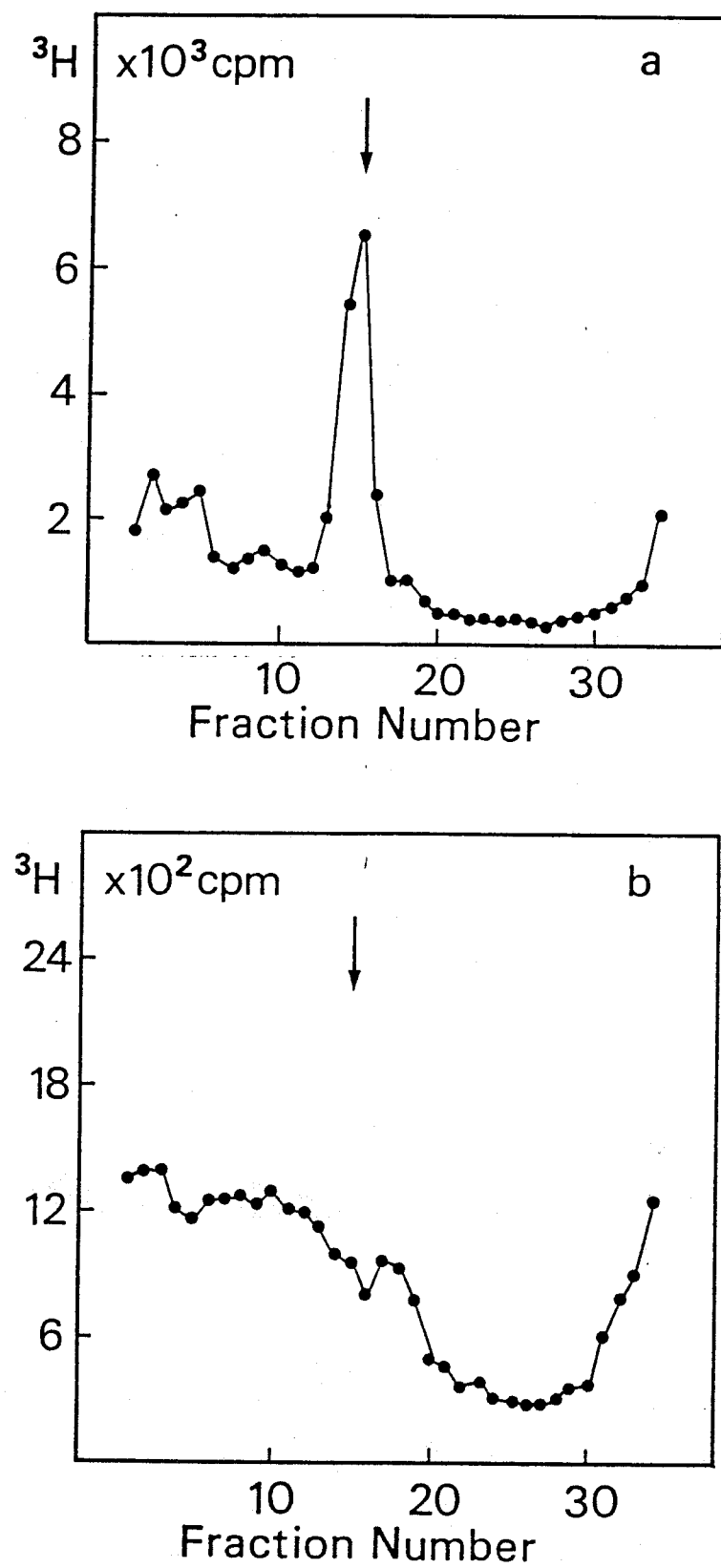
Analysis of intracellular DNAs of T7 *ts2.5* infected cells

Figure 10.

UV sensitivity of ssb mutants

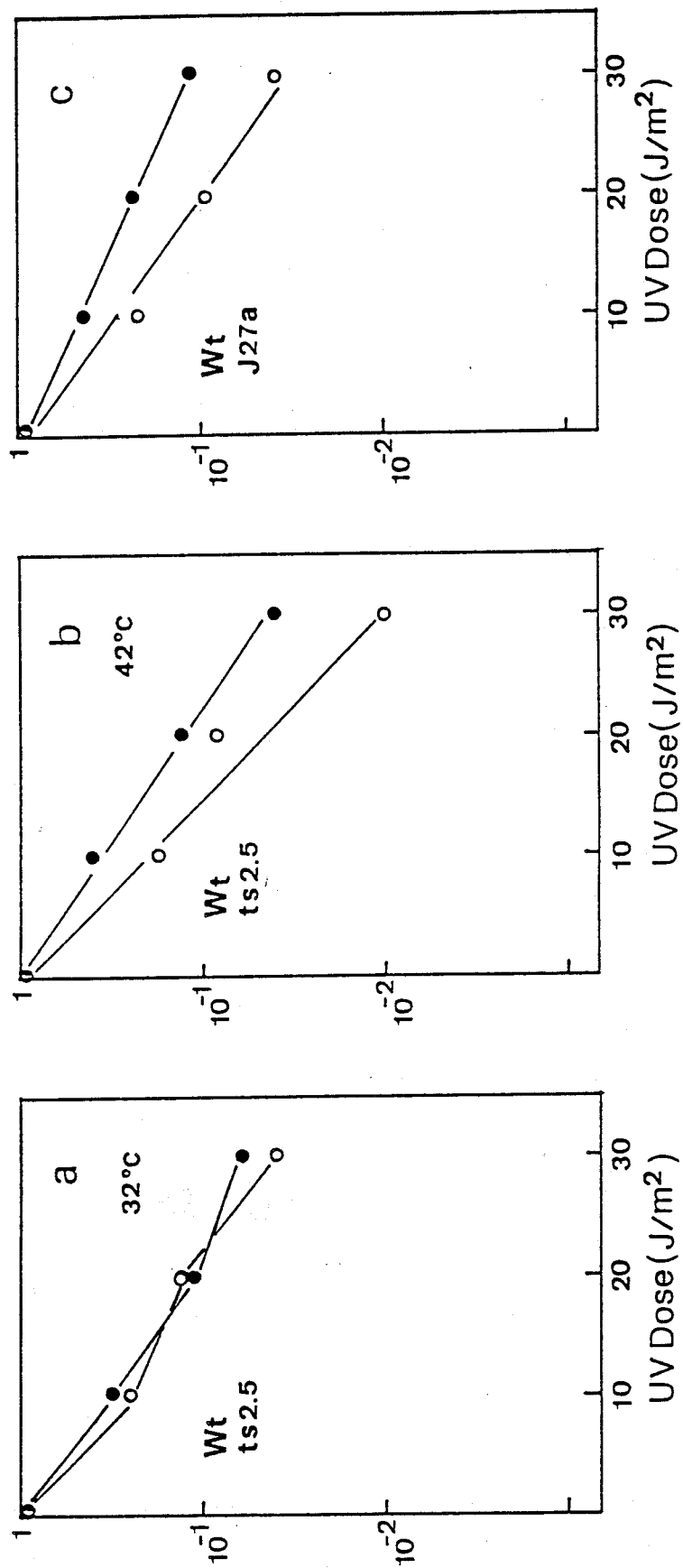


Figure 11.

Recentrifugation profile of T7 DNA

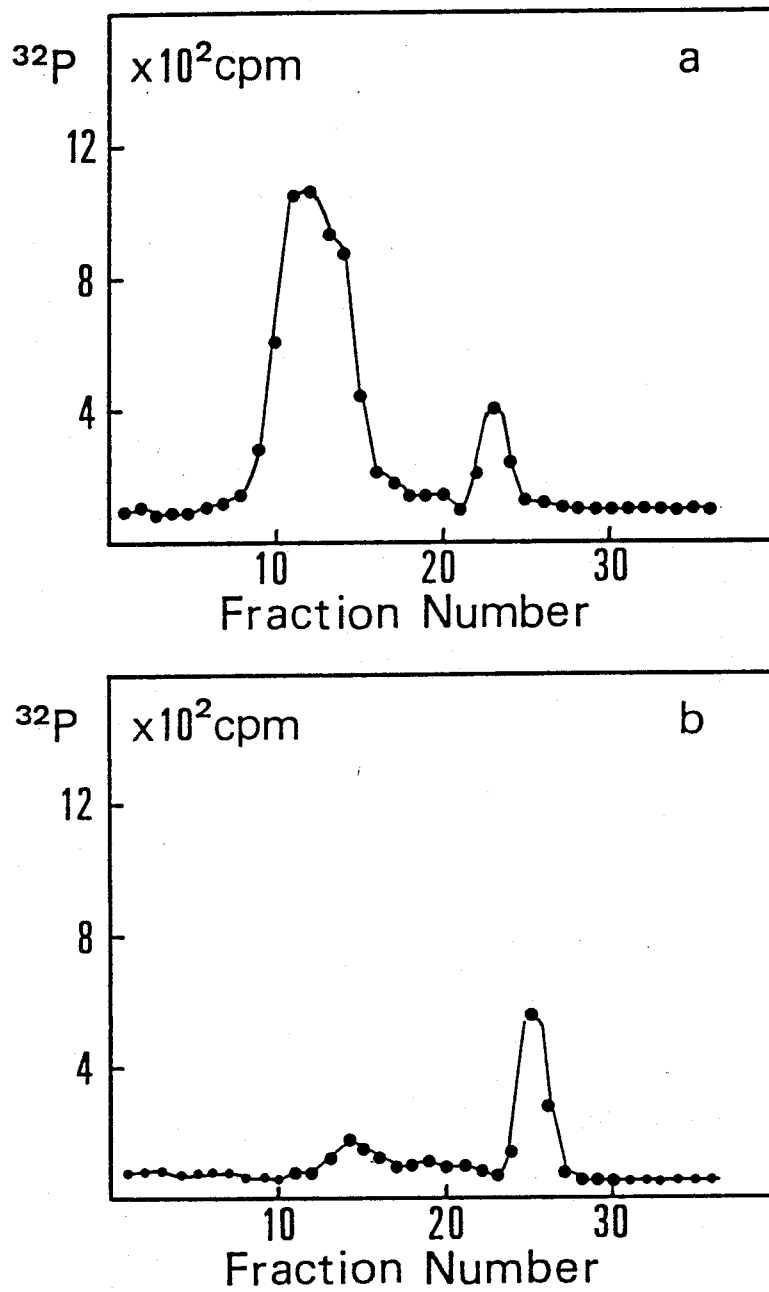


Figure 12.

CHAPTER I

CHAPTER II

Regulation of gene 2.5 expression

CHAPTER II

Abstract

Gene 2.5 of T7 phage locates in the class II transcriptional legion of T7 genetic map, and the mRNA that is transcribed from ϕ 2.5 promoter covers mRNAs for gene 2.5, 2.8, 3 and 3.5.

Gene 3.5 product is lysozyme and is necessary for DNA replication. T7 lysozyme can bind to T7 RNA polymerase and inhibit its activity.

I suppose that a certain mechanism of regulation for ϕ 2.5 operon should exist, otherwise, the expression of gene 3.5 at early stage of infection represses the expression of other gene products.

The products of gene 2.5, 3 and 3.5 are ssb, endonuclease and lysozyme respectively, but the activity of gene 2.8 is not known yet. So I attempt to isolate the mutants of gene 2.8 by *in vitro* mutagenesis. Two mutants were isolated, and *am2.8-1* bore the amber mutation near the N-terminal of the peptide and *am2.8-2* had the amber mutation near the C-terminal of the peptide.

The plating efficiency of *am2.8-1* was partially recovered by the presence of cloned gene 1 which was the gene for T7 RNA polymerase, and *am2.8-2* could not complement with *op1* and *am3*.

I also discussed the homology of gene 2.8 with gene 7.7, and the mechanism of gene expression of T7 phage.

Introduction

The gene expression of bacteriophage T7 was tightly regulated by several mechanisms. The class I genes are transcribed by host RNA polymerase, and then T7 RNA polymerase that is transcribed by host RNA polymerase transcribes class II and III genes. In the early stage of expression of class II genes, host RNA polymerase is inactivated by gene 0.7 product (class I) and gene 2 product (class II). The switching of class II and class III gene expression is seemed to be controlled by T7 lysozyme (gene 3.5 product) (Moffat and Studier 1987). T7 lysozyme tightly binds to T7 RNA polymerase and reduced the polymerase activity. By this modification the transcription of T7 genes is shifted from class II promoter to the stronger class III promoters. The expression of T7 lysozyme has to be controlled, since the expression of lysozyme in the early stage of infection causes to shut off the transcription from class II promoters prematurely.

T7 lysozyme mRNA is transcribed from $\phi 2.5$ promoter. In the case of bacteriophage T4, *regA* gene products bind the control region of several mRNAs, and represses the gene expression at translational level (Adari et.al. 1985, Winter et.al. 1987). T4 *regA* protein is not similar to T7 gene 2.8 protein at amino acids sequence level but both are very basic peptides. (pI of *regA* protein is 9.8 and that of gene 2.8 protein is 9.1).

On one hand, T4 gene 32 product binds its own ribosomal initiation site (translational operator) of mRNA and thus controls its own synthesis (Hippels et.al. 1983). On the analogy of the case of T4 phage, I suppose that some translational repressors exist in T7 phage. Gene 2.8 product is the potential candidate for the repressor.

Furthermore, by the homology search, I found that T7 gene 7.7 product resembled the gene 2.8 product at amino acids level. This is interesting finding to speculate the functions of these genes.

CHAPTER II

Materials and Methods

Buffers and Media:

All buffers and Media which were used in this CHAPTER were described in Materials and Methods of CHAPTER I. All of radioisotopes were purchased from Amersham, Japan.

Enzymes:

Restriction enzymes, T4 polynucleotide kinase and ligation kit were from Takara. Oligonucleotide-directed mutagenesis system (ver.2) was from Amersham. T7 sequencing kit was from Pharmacia.

DNA and Oligonucleotides:

M13mp18 DNA was purchased from Takara. Oligonucleoties (5'-GACTTCCTA-GGGGTGTTGGG-3') for *am2.8-1* and (5'-GTCTCCCAGTAGACTATTTG-3') for *am2.8-2* for *in vitro* mutagenesis were purchased from Takara.

Bacterial plasmid and phage strains:

All bacterial and phage strains used in this CHAPTER were shown in Materials and Methods of CHAPTER I. Plasmid pTI1219 (Figure 15.) is kindly gifted from Dr. T. Itoh. Plasmid M13mp18bb is described in CHAPTER I.

Isolation of gene 2.8 mutants by oligonucleotide-directed mutagenesis:

The strategy of isolation of gene 2.8 amber mutants is same as that of gene 2.5 in CHAPTER I. The method is described in Materials and Methods of CHAPTER I.

Complementation tests:

Complementation test between mutant phages was carried out by the method described in Studier (1969). Complementation by plasmid carrying gene *l* was follows; Overnight culture of *E.coli* carrying the pTI1219 plasmid, was diluted by 50 fold into T-broth, and grown to the density of 1×10^8 cells/ml and IPTG*⁵ was added to the culture to the final concentration of 10mM. After 1 hr, the culture was used for phage titration.

Homology search between data base and product of gene 2.8:

Homology search was carried out using PROSRCH program (MKI).

Results

Isolation of gene 2.8 amber mutants:

The same strategy was used for isolation of gene 2.8 amber mutants as gene 2.5 mutants isolation (see Materials and Methods of CHAPTER I.). One was designed to locate at Ser16 (*am2.8-1* mutation), and another, at Gln123 (*am2.8-2* mutation). The presence of mutation sites of *am2.8-1* and *am2.8-2* was confirmed by restriction enzyme digestion. Both mutations were designed to create a sensitive site for *EcoT14I* or *AccI*. The DNAs of mutants phages were isolated and digested by *BcII* and *BglII*, and followed agarose gel electrophoresis then 3204bp fragments were isolated.

The new restriction site created by introducing the mutation within *BcII*-*BglII* fragments was checked by *EcoT14I* (for *am2.8-1*) or *AccI* (for *am2.8-2*) digestion. The mutation sites and the diagram of mutants are shown in Figure 13. The plating efficiency of *am2.8-1* or *am2.8-2* was shown in Table 6. The ratio of the plating efficiency on su^- to that on su^+ for both mutants was same as other T7 amber mutants (ca. 10^{-6} - 10^{-5}).

Complementation tests:

The complementation test was carried out between the mutant phages which were listed in Table 5 . and the *am2.8-1* or *am2.8-2* mutant phage (Table 7). The results were follows: mutant *am2.8-1* made a clear spot with all other mutants except *am1* (gene 1-19), but *am2.8-2* did not make a spot with *am2.5-2* or *op1* or *am3*.

Suppression for the defect of *am2.8-1* by the presence of the plasmid carrying gene 1 RNA polymerase:

Mutation *am2.8-1* was not complemented with *am1*, the complementation by plasmid carrying gene 1 was examined.

The plating efficiency of *am1* phage was almost one on su^- bacterial carrying the plasmid with the gene 1 RNA polymerase. Similarly phage *am2.8-1* could restore the

plaque forming ability to 2×10^{-3} on this su^- bacteria carrying the plasmid with gene *I* strain, although its plaque size is very small. In the case of *am2.8-2*, the plasmid carrying gene *I* did not affect its plating efficiency. (Table 6.). This shows that the defect of the growth of *am2.8-1* could be suppressed by the presence of the enough amount of T7 RNA polymerase.

Homology of gene 2.8 product with gene 7.7 product:

The homology search of amino acids sequence of gene 2.8 peptide by the PROSRCH program (MKI) revealed that out of 10,000 peptides, one peptide, T7 gene 7.7 product was very similar to this peptide. Sixty five amino acids residues of gene 2.8 products matches to those of gene 7.7 peptide (Figure 14). Gene 7.7 product has 130 amino acids and is basic peptide ($pI=9.4$), and gene 2.8 product has 139 amino acids and its $pI=9.1$. Those facts, suggest that both peptides play a same role in the T7 phage. The gene 2.8 locates in the early site of the class II genes and the gene 7.7 locates in the early site of the class III genes. It is likely that the gene 2.8 controls the expression of the class II genes and the gene 7.7 controls the expression of the class III genes.

Discussion

The two amber mutants of gene 2.8 were isolated by *in vitro* mutagenesis (Figure 13). The *am2.8-1* mutation was designed for the gene 2.8 to create a short 15 amino acids polypeptide (10% size of the whole 2.8 protein), and the *am2.8-2* mutation to create a 122 amino acids polypeptide (88% size of the whole). These mutants showed anomalous properties in the complementation test. The mutant *am2.8-1* could not be complemented with *am1*, whereas the mutant *am2.8-2* was not complemented with *am2.5* or *am3*. However *am2.8-1* recovered the plating efficiency on the restrictive host carrying the gene 1 on a multicopy plasmid, pTI1219. These means that the overproduction of T7 RNA polymerase could overcome an almost complete defectiveness in the gene 2.8.

On the other hand, the overproduction of the product of the gene 3.5, lysozyme, is known to inhibit T7 phage growth severely. This growth inhibition can be overcome by the mutations within the gene 1 (Moffatt and Studier, 1987). Further, a mutation in the gene 1 could relieve the defectiveness of *am2.8-1* (data not shown). Those facts suggested that T7 *am2.8-1* mutation may lead to overproduction of gene 3.5 lysozyme, which inhibits the T7 RNA polymerase activity and that the overproduction of gene 1 RNA polymerase by introduction of the plasmid pTI1219 into the host cells, could recover phage growth partially. The introduction of the plasmid pTI1219 in the host, however, supported neither the growth of T7 *am2.8-2*, nor complementation of *am2.8-2* with *am2.5* and *am3*. This suggested that T7 *am2.8-2* could not make gene 2.5 and gene 3 products normally.

Considering these results, I hypothesized for the function of T7 gene 2.8 product that the gene 2.8 product negatively controls the expression of gene 2.5, 3 and 3.5 which are transcribed as single mRNA, by binding to their mRNA. In the absence of gene 2.8 function by mutation *am2.8-1*, gene 3.5 (perhaps also 2.5 and 3) product will be overproduced by the absence of the negative control to transcript covered with the gene

2.5, 3 and 3.5 genes. However the *am2.8-2* mutant product probably have an anomalous binding activity and will repress the translation of gene 2.5 and 3 (perhaps also 3.5) tightly (Figure 16).

By their analysis of the synthesized DNA from R9 infected cells in the sucrose gradient centrifugation, North and Molineux (1980) reported on the T7 mutant *R9*, that was defective in early DNA synthesis, and has a mutation between the gene 2 and the gene 3. The profiles of their centrifugation patterns were similar to those of the DNA isolated from 3.5 defective phage infected cells. The *R9* mutation probably in the gene 2.8, and has a similar characteristics to the mutation *am2.8-2*. The mutation may inhibits the expression of gene 3.5. Therefore, the phenotype of the mutant resembles the gene 3.5 mutant.

A rifampicin-resistant RNA polymerase mutant, *RpoB26* of *E.coli*, causes the inhibition of the growth of T7 phage, and the inhibition is due to a higher rate of termination of transcription by the mutant host RNA polymerase before gene 1 T7 RNA polymerase is synthesized at an enough amount. Yeats *et al.* (1981) isolated the T7 mutant which could grow on *RpoB26*. mutant The isolated mutant *HS36* was found to carry a deletion affecting a T7 gene 2.5 and gene 2.8. From this result they suggested that T7 gene 2.5 product (ssb protein) may stimulate the termination of transcription, and that the inactivation of the gene 2.5 product may restore the normal level of the transcription termination. They also suggested that an alternative possibility: the altered gene 2.8 protein may increase the expression level of T7 RNA polymerase indirectly as mentioned above. Figure 15 shows the comparison of DNA sequences of initiation sites for translation of gene 2.8 and gene 3.5. In both sequences, four "AAA" sequences exist at the upstream region of initiation codon. In the case of T4 phage, same feature of sequence is reported in gene 32, and gene 32 protein is known to bind those regions, and control its expression. Furthermore, T4 phage has another regulation factor, *regA* product. The *regA* protein controls several T4 early genes negatively. The *regA* protein binds specifically to target site near the initiation codon (AUG) of mRNA. and represses the gene expression at the translational level (Adari

CHAPTER II

et.al. 1985; Winter et.al. 1987). If a similar kind of the regulation mechanism of gene expression of T4 phage are available in T7 phage, the following model could be proposed for the gene expression in early stage of T7 phage infection.

When T7 phage DNA is injected into the bacterial cell, by the phage infection, bacterial RNA polymerase start to transcribe the class I genes, and genes 0.3-1.3 products will be produced. The gene 0.7 protein produced will inhibit bacterial RNA polymerase activity, and then T7 RNA polymerase will start to transcribe the class II genes. There are seven promoters in class II genes, and genes 2.5-3.5 are transcribed from former three promoters ($\phi 1.5$, $\phi 1.6$ and $\phi 2.5$). Gene 3.5 protein will tightly bind to T7 RNA polymerase and then the activity of T7 RNA polymerase will be reduced, so that the transcription of T7 genes will be shifted from the class II promoter to the stronger class III promoters. In this process of transcription shift, the expression of gene 3.5 must be repressed until the other class II genes are transcribed sufficiently. The gene 2.8 product may control the expression of the gene 3.5. To confirm this hypothesis, the gene 2.8 protein must be purified and must be examined its binding of this protein to the specific transcript covering the gene 2.5-3.5 directly.

T7 gene 7.7 product was found to be very similar to the gene 2.8 product at amino acids level in this work (Figure 14). Since the gene 2.8 mutants are lethal on the su⁻ hosts, the gene 7.7 product cannot complement the defect of the gene 2.8 mutants. However, it probably plays the same role in the T7 phage development. For example, they mutually have different specificity to a binding site on mRNA, and they may regulate the translation of the the different transcripts. It will be important to isolate the gene 7.7 mutants and to characterize them for understanding of the role of gene 2.8 protein.

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Legends

Table 6.

Plating efficiency of *am2.8-1* and *am2.8-2*

Table 7.

Complementation test between amber phages

(-) means that the plaque did not form. (+) means that the plaque formed.

Figure 13.

Mutation sites and diagram of gene 2.8 mutants

(a) mutation sites of gene 2.8 mutants

(b) diagram of mutants

Figure 14.

Sequence homology of amino acids between gene 2.8 and gene 7.7

Asterisk(*) shows both amino acids of gene 2.8 and 7.7 are identical, and vertical bar(|) shows that both amino acids are similar.

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Figure 15.

Comparison of initiation sites for synthesis of proteins

The sequences of protein initiation sites of gene 2.5 and gene 3.5 was compared. Four 'AAA' sequences exist in the upstream of ATG codon.

Figure 16.

Model for regulation of gene expression by 2.8 protein

- (a) In the case of *wild type* T7 phage: The active 2.8 protein modulates normally the expression of genes 2.5, 3 and 3.5
- (b) In the case of T7 *am2.8-1* phage: The *am2.8-1* mutant protein is defective to modulate the normal expression of gene 2.5, 3 and 3.5, so the overexpression of them occur.
- (c) In the case of T7 *am2.8-2* phage: The *am2.8-2* mutant protein represses tightly the expression of gene 2.5, 3 and 3.5, so the few amounts of them express.

Tables

Table 6.

Plating efficiency of *am2.8-1* and *am2.8-2*

	594	WD001	WD002	WD003	594(pTI1219)
<i>am2.8-1</i>	7.2×10^{-6}	1	-	-	2×10^{-3} (sp)
<i>am2.8-2</i>	2.7×10^{-6}	-	-	1	1.1×10^{-6}
<i>am1</i>	4.3×10^{-7}	-	1	-	0.36
<i>am3</i>	5.5×10^{-6}	-	1	-	2.3×10^{-6}

-:not tested, sp:small plaque

Table 7.
Complementation test between amber phages

	<i>am1</i>	<i>am2</i>	<i>am2.5</i>	<i>am2.8-1</i>	<i>am2.8-2</i>	<i>am3</i>	<i>am3.5</i>	<i>am4-am19</i>
<i>am2.8-1</i>	-	+	+	-	+	+	NT	+
<i>am2.8-2</i>	+	+	-	+	-	-	NT	+

NT: not tested

Figures

Mutation sites and diagram of gene 2.8 mutants

(a) mutation sites of gene 2.8 mutants

(*EcoT14I*)

1 10 AG (Ser-amber)
 GTGGAAGTGC GGGAGAAAATCCTTGAGCGAATCAAGGTGACTTCCTCTGGGTGTTGGGAG
 MetGluLeuArgGluLysIleLeuGluArgIleLysValThrSerSerGlyCysTrpGlu
***am2.8-1

21 30 40
 TGGCAGGGCGCTACGAACAATAAAGGGTACGGGCAGGTGTGGTGCAGCAATACCGGAAAG
 TrpGlnGlyAlaThrAsnAsnLysGlyTyrGlyGlnValTrpCysSerAsnThrGlyLys

41 50 60
 GTTGTCTACTGTCATCGCGTAATGTCTAATGCTCCGAAAGGTTCTACCGTCCTGCACTCC
 ValValTyrCysHisArgValMetSerAsnAlaProLysGlySerThrValLeuHisSer

61 70 80
 TGTGATAATCCATTATGTTGTAACCCTGAACACCTATCCATAGGAACTCCAAAAGAGAAC
 CysAspAsnProLeuCysCysAsnProGluHisLeuSerIleGlyThrProLysGluAsn

81 90 100
 TCCACTGACATGGTAAATAAGGGTCGCTCACACAAGGGGTATAAACTTTCAGACGAAGAC
 SerThrAspMetValAsnLysGlyArgSerHisLysGlyTyrLysLeuSerAspGluAsp

101 110 120
 GTAATGGCAATCATGGAGTCCAGCGAGTCCAATGTATCCTTAGCTCGCACCTATGGTGTC
 ValMetAlaIleMetGluSerSerGluSerAsnValSerLeuAlaArgThrTyrGlyVal
 (*AccI*)

121 130 139
 TCCCAACAGACTATTTGTGATATACGCAAAGGGAGGCGACATGGCAGGTTACGGCGCTAA
 SerGlnGlnThrIleCysAspIleArgLysGlyArgArgHisGlyArgLeuArgArg***
 ***am2.8-2

Figure 13.

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(b) diagram of mutants

Diagram of gene 2.8 mutants

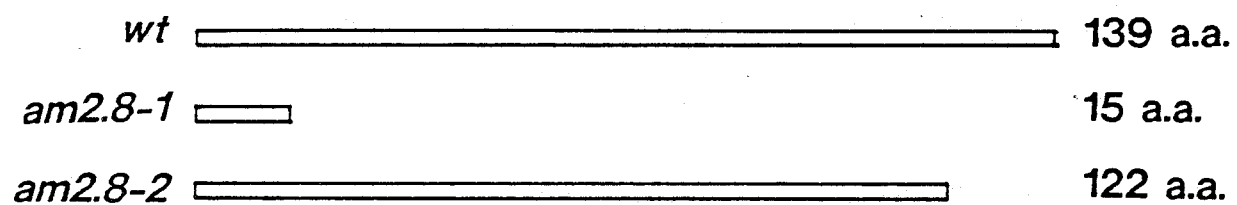


Figure 13 (cont.)

Sequence homology of amino acids between gene 2.8 and gene 7.7

T7 gene 2.8 (1-139) MW 15,617
 T7 gene 7.7 (1-130) MW 14,737

```

2.8  MELREKILERIKVTSSGCWEWQGATNNKGYGQVWCSNTGKVVYCHR-V--MS-NA-PK
      * * * * * * * * * * * * * * * * * * * * * *
7.7  -----MEDCIEWTGGVNSKGYGRKW-VN-GKLVTPhrHIYEETyGPVPT

GSTVLHSCDNPLCCNPEHLSIGTPKENSTDMVNKGRSHKG--Y--KLSDedVMaIMES
*  * | * * * * * * * * * * * * * * * * * * * * * * * * *
GIVVMHICDNPRCYNIKHLTLGTPKDnSEdMvTKGRQAKGEELSKKLtESdVLAIrSS

SESNVSLARTYGVSQQTICDI--RKGRRHGRLRR
* | ** * * * * * * * * * * * * * * * *
TLShRSLGELyGVSQStITrILQrKtWRHI----
```

*:identical, |:similar

Figure 14.

CHAPTER II

Comparison of initiation sites for synthesis of proteins

gene 2.5

```
                -10      1
CTCGTGTGCGTCCTTGTGTAGCACCGAAGTAATACGACTCACTATTAGGGAAGACTCCCT
CTGAGAAACCAAACGAAACCTAAAGGAGATTAAACATTATGGCTAAGAAGATTTTCACCTC
                ***
```

gene 3.5

```
TTAAGTTCGCTGATAAACTGATACCTGCTGAGTGGATAAAGGAACCCAAGAAGGAGGTCC
CCTTTGATAGATTAAAAAGGAAAGGAGGAAAGAAATAATGGCTCGTGTACAGTTTAAACA
                ***
```

Figure 15.

Model for regulation of gene expression by 2.8 protein

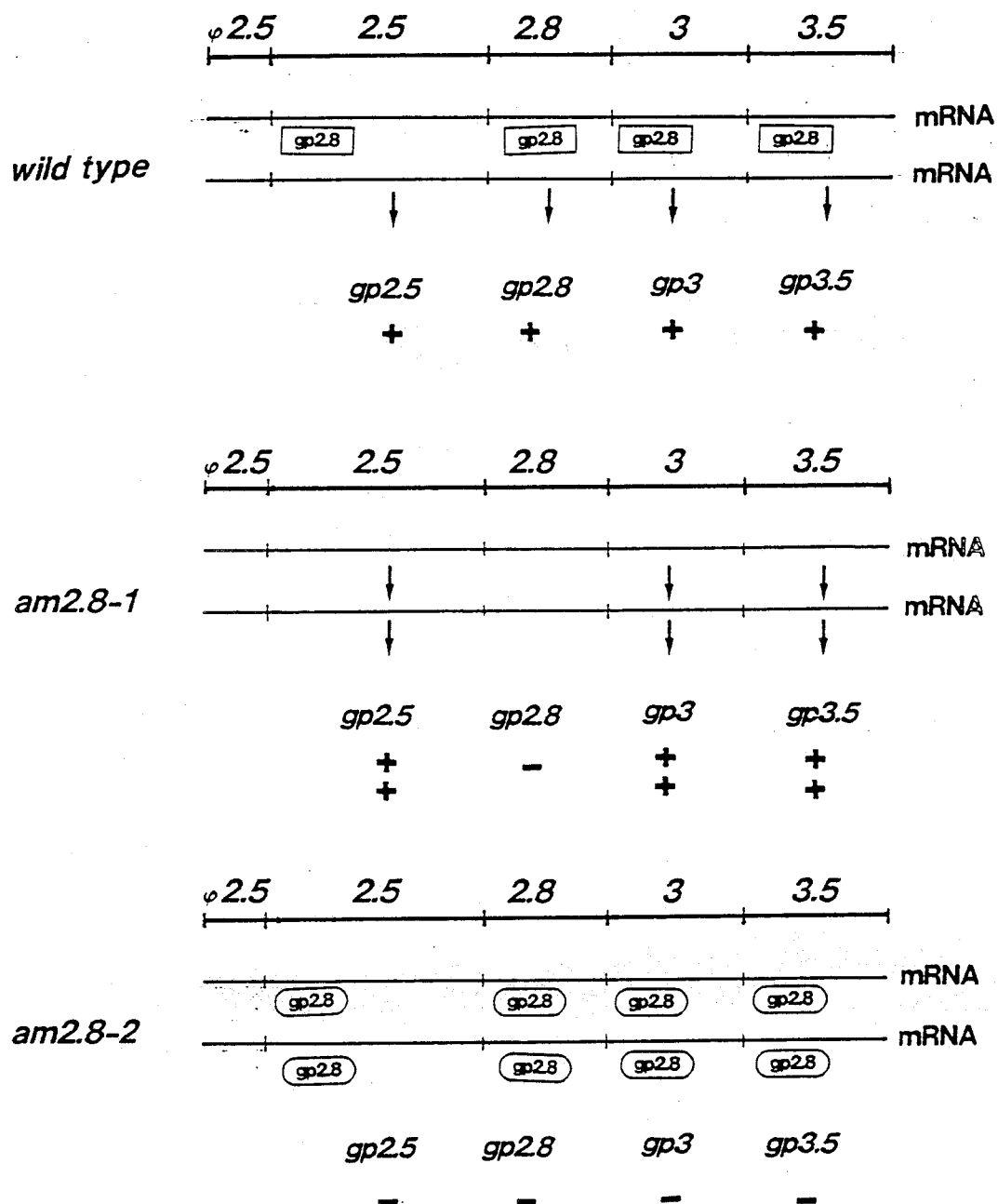


Figure 16.

CHAPTER II

CHAPTER III

Isolation and characterization of gene *17.5* mutant

CHAPTER III

Abstract

In the course of isolation of new mutants in gene 2.5, I obtained a mutant of the new gene, which was complemented with all known mutants perfectly, but partially complemented with *opl* partially. This new mutant was mapped between the gene 17 and the gene 18, so I named it the gene 17.5 according to the rule of the naming of T7 phage mutant. The DNA extracted from this mutant phage infected cells was examined. The length of the DNA was one fifth size of the monomer T7 DNA.

This result suggests that the processing site is determined by gene 17.5 product, and in the absence of the product, the imprecise cutting is occurred

Introduction

The new mutant of T7 phage was isolated by chance when I attempt to isolate the mutants of the gene 2.5. That was selected as the phage which could complement with all T7 mutants except *op1*. However, the genetic cross experiments with *am2* and *am3* showed that the mutation (#26) was not located near the gene 2.5. The location of the mutation was determined by the genetic cross with several T7 amber mutants. Those results showed that the mutation was located between the gene 17 and the gene 18. so it was named the gene 17.5. The products of the gene 18 and gene 19, which were located near this mutant, participate to the processing of the concatemers, so the DNA extracted from gene 17.5 phage infected cells was analyzed.

Results and Discussion

Characterization of gene *17.5*

The Materials and Methods used in this CHAPTER were described in previous CHAPTERS. The results of two or three factor cross of this mutant (#26) with *am17* and *am18* were shown in Figure 17. The results showed that the new mutation located between gene *17* and gene *18*. From the nucleotide sequence of T7 phage (Dann and Studier 1983), gene *17.5* should correspond to the gene identified by this mutant, and should encode a protein with molecular weight of 7391 Dalton. The gene *17.5* had been a predicted one from the analysis of nucleotide sequence of T7 DNA, this isolation of the mutant proved its existence.

In the case of bacteriophage T3, the gene *17.5* encoded lysozyme (lys) (Miyazaki, Ryo, Fujisawa and Minagawa 1978). To test a possibility that the gene *17.5* product of T7 phage is a lysozyme gene, I carried out one-step growth experiments using 594 as a host. The result shows that any difference in the eclipse period and burst size was not observed (data not shown). The sedimentation pattern of DNA synthesized after T7 gene *17.5* mutant infection was examined by sucrose density gradient centrifugation. The results were shown in Figure 18. As comparison, DNA extracted from the gene *18* mutant infected 594 cells as well as DNA from wild type phage infected cells were analyzed similarly. As expected, DNA from gene *18* mutant infected cells was sedimented as concatemers, and DNA from *wild type* infected cells, as monomers (Figure 18-(c), Figure 7-(a)). On the other hand, DNA from the gene *17.5* mutant phage infected 594 cells was one fifth size of monomer length (Figure 18-(a)). When DNA was extract from the gene *17.5* infected 594^{su⁺2} cells, the sedimentation pattern is similar to that of wild type (Figure 18-(b)). The participation of the product of gene *17.5* to the maturation was not obvious, but one possibility is that the processing site was determined by gene *17.5* product, and in the absence of the product the imprecise cutting was occurred.

However the possibility that the DNA in the gene 17.5 infected cells were more sensitive to bacterial or phage nucleases than that in *wild type* infected cells, so it was digested randomly. The recombination frequency of gene 17.5 mutant was also measured, but apparent difference from the frequency of the wild type was not detected. The relationship between gene 2.5 and gene 17.5 is not known now yet, but gene 17.5, 18, 19 and 2.5 products may function together in the course of processing of concatemer DNA.

CHAPTER III

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Legends

Figure 17.

Genetic mapping of #26.

Cells of *E.coli* 594 were grown at 32°C in T-broth to 2×10^8 cells/ml. Cells were infected with two mutants phages at multiplicity of infection of 10 each. They were diluted by 10^4 fold after 5 min of infection. The cells were incubated further for 60 min and chloroform was added to the cells. Map distance (A) was given as following equation:

$$A = 2 \times \frac{\text{plaques on nonpermissive host}}{\text{plaques on permissive host}}$$

Figure 18.

Analysis of intracellular DNAs of T7 #26 infected cells

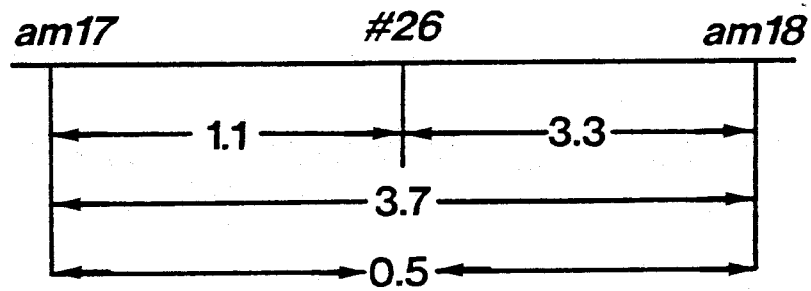
Method was described in legends in Figure 10 of CHAPTER I.

(a):#26 in 594 (b):#26 in 594^{su+2} (c):*am18* on 594

The arrow indicates the position of monomer T7 DNA.

Figures

Genetic mapping of #26.



cross	recombination freq
#26 x am17	1.1%
#26 x am18	3.3%
am17 x am18	3.7%
#26 x am17am18	0.5%

Figure 17.

Analysis of intracellular DNAs of T7 #26 infected cells

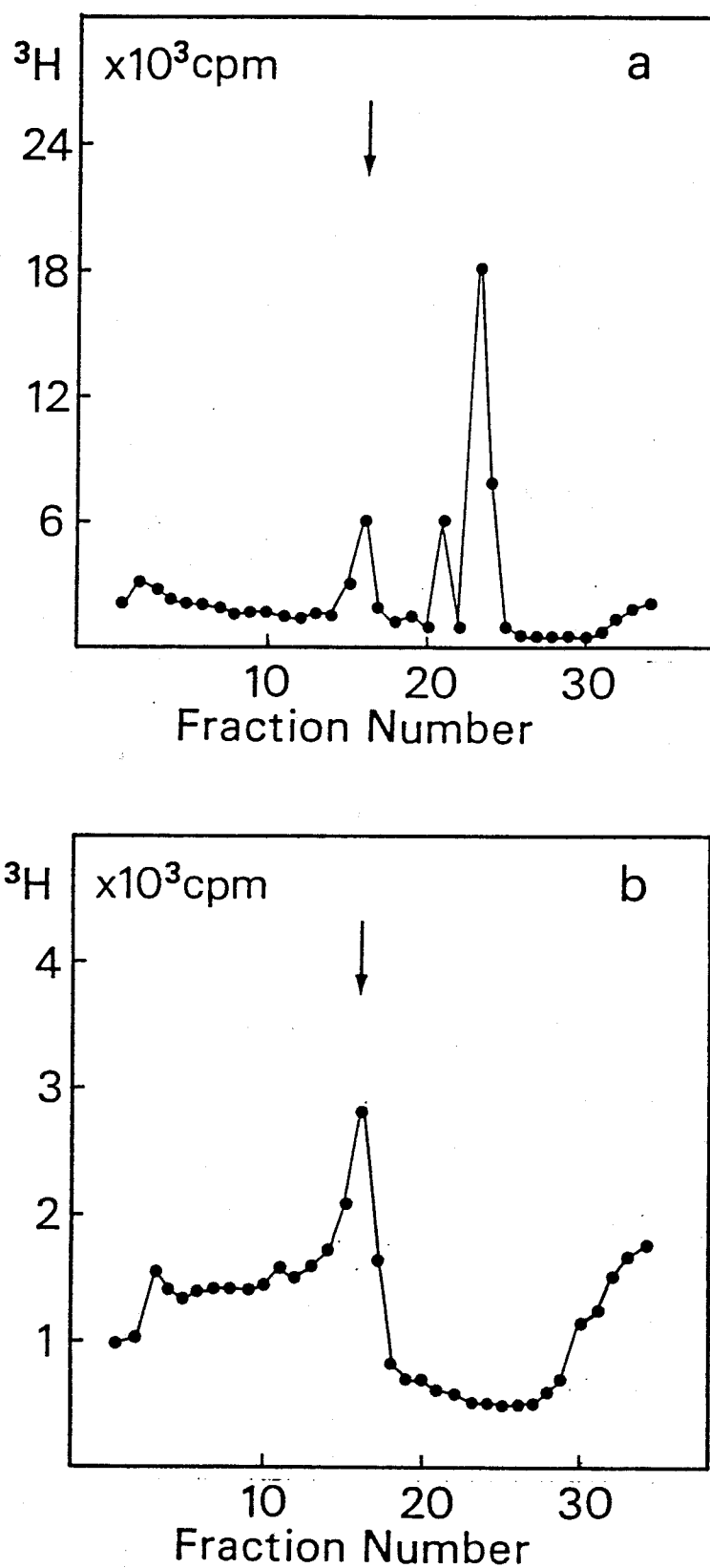


Figure 18.

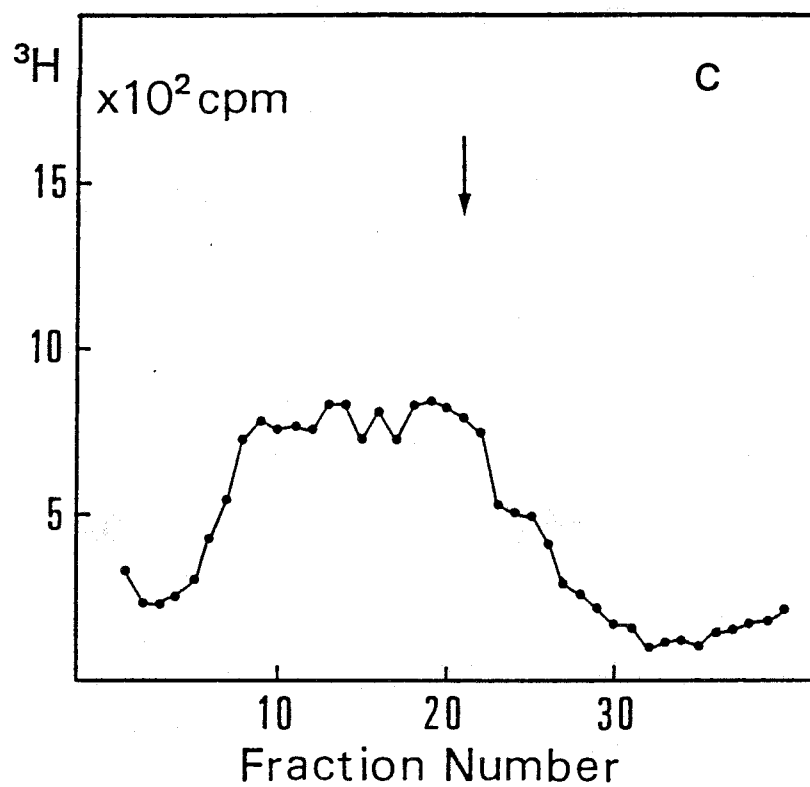


Figure 18 (cont.)

Conclusion

In this study, I showed the followings about T7 gene 2.5. (A) T7 phage single-stranded DNA-binding protein is essential for phage growth, especially for DNA recombination and DNA synthesis. The null mutant of T7 ssb gene (*am2.5-1*) could not synthesize DNA and was defective in recombination on the *su-* host, whether or not the host has wild type ssb gene. (B) T7 ssb probably interacts physically with several T7 gene products. The first isolated mutant in T7 gene 2.5 *up2*, had two additional mutations outside the gene 2.5. One of the mutations located in the gene 6, and the other mutation located in the gene 18. It was reported that T7 ssb enhanced T7 exonuclease activity (1,2). Moreover the interaction of gene 2.5 protein with the T7 DNA polymerase and the gene 4 protein was reported previously (3,4). In this work, I showed that T7 ssb interacted with T7 exonuclease (gene 6 product) and the gene 18 product by genetical method. From this work, the following model for interaction between T7 ssb and T7 exonuclease or the gene 18 product can be proposed.

Wild type T7 ssb could enhance the activities of T7 exonuclease and the gene 18 product, but mutated ssb (*op1* protein) could not. However, when two additional By the mutations, *S⁻¹* and *S⁻²* occurred in this *op1* mutant, T7 exonuclease and the gene 18 product could be enhanced by bacterial ssb instead of T7 ssb.

The *S⁻¹* and *S⁻²* had no effect on other T7 ssb mutants (*am2.5-2* and *ts2.5*). Those results may be explained as follows: T7 phages, *am2.5-2* and *ts2.5* should loss the other activities which *op1* mutation still held, for example, the interaction with T7 DNA polymerase or the gene 4 primase, so *S⁻¹* and *S⁻²* mutations could not support that defectiveness. In 594 *ssb-1* and 594*ssb-113* strains, T7 *up2* phage could not grow because both phage and bacterial ssb were defective. (C) The expression of genes 2.5 and 3.5 is probably regulated by the gene 2.8 product. I also showed that T7 gene 2.5 and 3.5 was controlled by gene 2.8 product. The gene 2.8 product represses the expression of gene 2.5. One mutant, *am2.8-1*, which was expected to produce a small

15 amino acids polypeptide could not repress the expression of those genes, 2.5 and 3.5, and the gene 3.5 product was overproduced (Figure 16). The gene 3.5 product, lysozyme and inhibited the activity of T7 RNA polymerase (5). Another mutant *am2.8-2* which was expected to produce about 90% length of T7 gene 2.8 polypeptide and the product seems to inhibit more severely than wild type product. If so, the expression of the gene 2.5-3.5 will be repressed more tightly. This agreed with the fact that *am2.8-2* could not complement *opl*.

Citations for Conclusion

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Abbreviations

- 1) NTG; N-methyl-N'-nitro-N-nitrosoguanidine
- 2) TCA; Trichloro-acetic-acid
- 3) BU; 5-bromouracil
- 4) SLS; sodium N-lauroylsarcosinate
- 5) IPTG; isopropylthio- β -D-galactoside

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