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THE EARLY PROCESS OF GENETIC RECOMBINATION

——ROLE OF T7 DNA-BINDING PROTEIN——

By

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CONTENTS

I

INTRODUCTION

Using bacteriophage T7 as one of the simplest systems, my work started from the analysis of the early stage of general genetic recombination and has developed into the study about T7 DNA-binding protein which participates in the early stage of genetic recombination. As "genetic recombination" which means the rearrangement of genetic materials is occurred ubiquitously in all forms of life, it is one of the basic phenomena of life. Thus, the study of recombination is an important for the solution of the question, "What is life ?" and it will also reveal the dynamic aspects of "gene". Genetic recombination participates in evolution of organisms. A change of genetic material occurred in one organism is distributed by genetic recombination among the same species. The accumulation of changes of genetic material and the distribution of those by genetic recombination can make an original genotype to the various genotypes by the combination of changes of genetic material. For instance, if the three changes (A, B, and C) were occurred independently, 8 genotypes should be constructed (ie. no change, A, B, C, AB, BC, CA, ABC). The evolution can be explained by the repetition of this phenomenum. Therefore, the study about genetic recombination introduce us to the solution of the mechanism of evolution. Besides the contribution to the basic science, we will be able to construct useful genotypes for human welfare if the recombination mechanism would be understood.

Single-stranded DNA-binding protein which preferentially bind to single-stranded DNA has been isolated from many organisms and it has been appeared that this protein plays an important role in replication, recombination and repair. The functions of single-stranded DNA-binding protein have not been well known. The analysis of single-

stranded DNA-binding protein will reveal the mechanism of replication,

recombination and repair as it concerns to them.

At first, I outline some known evidences necessary for reading this

paper.

1) General genetic recombination

In general genetic recombination, exchange between homologous

DNA takes place anywhere along the length of the DNA molecules.

Genetic recombination occurs mostly in meiosis of Eukaryote and always

in Prokaryote and it gives the organism the variety and hence adaptation

and evolution by the mechanism described above.   Genetic recombination

also occurs between bacteriophages infecting high multiplicity.   In this

paper, for the analyses of genetic recombination bacteriophage T7 was

used because of its simplicity.   Genetic recombination has been studied

by the isolation and characterization of mutants defective in genetic

recombination and recently, enzymology of proteins involved in recombination

has joined to recombination research.   Recombination process can be

separated into two stages; formation of joint molecules between parental

DNAs and maturation of joint molecules to recombinant DNA.   In this

paper, the early stage (formation of joint molecules) is concerned.

In this section, two well-known systems, the genetic recombination of

Escherichia coli and bacteriophage T4 which represent two different

mechanisms in the early stage of genetic recombination, will be described

and that of bacteriophage T7 will be discussed in an another section.

In genetic recombination of E. coli, rec A protein (MW=38 kdaltons) is thought as a key enzyme. RecA protein assimilates single-stranded DNA to homologous double-stranded DNA (Shibata et al., 1979) and this activity seems to participate in the early stage of genetic recombination. Single-stranded DNA-binding protein (MW=74 kdaltons) and recBC protein (MW=268 kdaltons) also participate in genetic recombiantion. RecBC protein has two nuclease activities which are ATP-dependent exonuclease and partially ATP-dependent endonuclease (MacKay and Linn, 1974). If single-stranded DNA-binding protein is present, recBC protein works as an unwinding enzyme (MacKay and Linn, 1976). Single-stranded DNA-binding protein stimulates the assimilation of the single-satranded DNA to homologous duplex DNA catalyzed by recA protein (McEntee et al., 1980). RecF gene is also known to participate in recombination that recBC protein is not concerned with but its function have not been elucidated (Horii and Clark, 1973). Whole mechanism of genetic recombination in E. coli is, therefore, obscure.

In the early stage of genetic recombination in bacteriophage T4, complementary single-stranded DNA region created by gene 46/47 exonuclease (MW=35 kdaltons) is renaturated by gene 32 DNA-binding protein (MW=35 kdaltons) (Broker and Lehman, 1971). Therefore, in contrast with the case of E. coli the creature of single-stranded DNA is essential for genetic recombination. Similar mechanism takes place in bacteriophage T7.

As described above, there is two types in the early stage of genetic recombination; single-stranded assimilation (E. coli) and renaturation of complementary single-stranded DNA created by nuclease (T4 phage).

2) Bacteriophage T7

Bacteriophage T7 is a virulent phage with 40 kb DNA.   About 30
genes are known and 19 of them are essential for phage growth.   The
genes are numbered from 1 to 20, going from left to right on the map
and are called as a number (Fig. I-1) (Studier, 1969, 1972).
Genes (Class I) that code for early functions in phage growth, are
situated at the left end of the map and are transcribed early by E. coli
RNA polymerase whereas the genes (Class III) of phage morphogenesis
are transcribed later by T7 RNA polymerase (gene 1) and are located in
the right half.   The genes (Class II) required for DNA replication and
recombination are clustered in the middle region which is transcribed by
T7 RNA polymerase in early late period.   Transcription is exclusively
from left to right end . (Hausmann, 1976).

Nine of the T7 genes are required for replication; T7 RNA polymerase
(gene 1), T7 ligase (gene 1.3), E. coli RNA polymerase inhibitor (gene 2),
T7 DNA-binding protein (gene 2.5), T7 endonuclease I (gene 3), T7 lysozyme
(gene 3.5), T7 primase (gene 4), T7 DNA polymerase (gene 5) and T7 exonuclease
(gene 6).   And six of them are also required for genetic recombination;
T7 ligase (gene 1.3), T7 DNA-binding protein (gene 2.5), T7 endonucleaseII
(gene 3), T7 primase (gene 4), T7 DNA polymerase (gene 5) and T7 exonuclease
(gene 6).   Roles of them in replication and recombination will be described
later.

3) DNA replication of bacteriophage T7

Nine proteins are required for T7 DNA replication and three proteins
of them (T7 primase, T7 DNA polymerase, T7 DNA-binding protein) directly
participate in replication of T7 DNA.   T7 primase coded by gene 4
(MW=58 kdaltons) has dual functions; helicase activity and RNA priming

```
           GENE        FUNCTION

         ┌─ 0.3         abolish host restriction
     │   │  0.7         protein kinase
     │   │
  ClassI │
     │   │  1           RNA polymerase
     │   │
     ┌───┴─ 1.3         DNA ligase
 ─── │ ──── 2           inactive host RPase
     │      2.5         DNA-binding protein
     │      3           endonuclease I
     │      3.5         lysozyme
 ClassII    4           primase
     │
     │      5           DNA polymerase
     │
     ↓      6           5'-exonuclease
 ──────── 7             virion protein
     ↑
     │      8       ┐ head protein
     │      9       ┤ head assembly
     │     10       ┘
     │     11       ┐
     │             │ tail protein
     │     12      ┘
     │     13         virion protein
     │     14       ┐
 ClassIII  15       ┤ head protein
     │              │
     │     16
     │
     │     17         tail protein
     │
     │     18       ┐
     │             │ DNA maturation
     ↓     19      ┘
 ──────── 20           growth on λlysogen

         Right end
```

Figure I-1.   Genetic map of phage T7

activity (Scherzinger et al., 1977; Kolodner, R. and Richardson, 1977).

T7 DNA polymerase consisted of gene 5 protein (MW=87 kdaltons) and

E. coli thioredoxin (MW=12 kdaltons), uses the primer synthesized by

gene 4 protein and elongates nucleotide chain (Sherzinger et al.,1977).

In vitro replicaiton of double-stranded DNA strictly requires both T7

primase and T7 DNA polymerase (Scherzinger and Klotz, 1975). T7 DNA-

binding protein (MW=25 kdaltons) stimulates T7 DNA polymerase activity

(Reuben and Gefter, 1973, 1974) and the double-stranded DNA replication

catalyzed by T7 DNA polymerase and T7 primase (Scherzinger and Klotz, 1975;

Richardson et al., 1978).   In vivo contribution of T7 DNA-binding

protein in T7 DNA replication was shown in this paper for the first time.

     Gene 2 protein (MW=8.5 kdaltons) which binds to E. coli RNA polymerase

and inhibits its activity (DeWyngaert and Hinkle, 1979) is required for

the synthesis of concatemeric T7 DNA in the late stage of T7 DNA replication

(Center, 1975).   T7 lysozyme (gene 3.5) (MW=13 kdaltons) seems to be

required for releasing newly synthesized T7 DNA from bacterial membrane

(Silberstein et al., 1975).   Both T7 endonuclease I (gene 3) (MW=14

kdaldons) and T7 exonuclease (gene 6) (MW=31 kdaltons) contribute to

the supply of the nucleotide precursors by the extensive breakdown

of host DNA (Sadowski and Kerr, 1970).   T7 exonuclease is also needed

for the removal of primer RNA (Shinozaki and Okazaki, 1978).

T7 ligase (MW= 40 kdaltons) (gene 1.3) is not essential for

T7 bacteriophage   growth as it can be complemented by

bacterial ligase (Masamune et al., 1971).   However the role of T7 RNA

polymerase(MW=107 kdaltons) in replication has not been clear, it may

stimulate the initiation of DNA replication by melting some portion of

DNA (Hinkle, 1980; Fischer and Hinkle, 1980).

4) Genetic recombination of bacteriophage T7

Bacteriophage T7 shows high recombination frequency.   Five genes
(genes, 1.3, 3, 4, 5, 6) have been known to be required for genetic
recombination of T7 phage (Powling and Knippers, 1974; Kerr and Sadowski,
1975).   In this paper, I show that T7 DNA-binding protein (gene 2.5) is
also required in addition to above five gene products.   Therefore, six
genes (1.3, 2.5, 3, 4, 5, 6) are required for genetic recombination of
T7 phage.

By the analyses of intermediate DNA molecules in genetic recombination,
Tsujimoto and Ogawa (1978) proposed the model of T7 genetic recombiantion.
Figure I-2 shows the model based on their idea and the results of this
paper.   Single-stranded gaps formed by T7 exonuclease (gene 6) allow
parental DNAs to interact with each other.   T7 DNA polymerase (gene 5)
stimulates the DNA interaction by forming a single-stranded structure
by repair synthesis or by 3'-exonucleotic activity.   T7 DNA-binding
protein (gene 2.5) stimulates the renaturation of complementary single-
stranded region created as above.   T7 endonuclease I (gene 3) acts on
branched intermediates and processes them to linear recombinant molecules
by cleaving single-stranded regions at the forks.   These linear
recombinant molecules with gaps or nicks are then converted to complete
recombinant molecules through the action of bacterial or phage DNA poly-
merase and ligase.

5) Single-stranded DNA-binding proteins

Single-stranded DNA-binding proteins have been isolated from many
organisms (Champoux, 1978).   In this section, three DNA-binding proteins,
T4 gene 32 protein, E. coli single-stranded DNA-binding protein and
T7 DNA-binding protein are described.   They lower the melting temperature

Parent DNA

5'-exonuclease
(gene 6)

Nicks ?

DNA polymerase
(gene 5)

T7 DNA-binding protein
(gene 2.5)

Endonuclease I
(gene 3)

Bacterial ligase

T7 ligase
(gene 1.3)

DNA polymerase I

T7 DNA polymerase
(gene 5)

Recombinant DNA
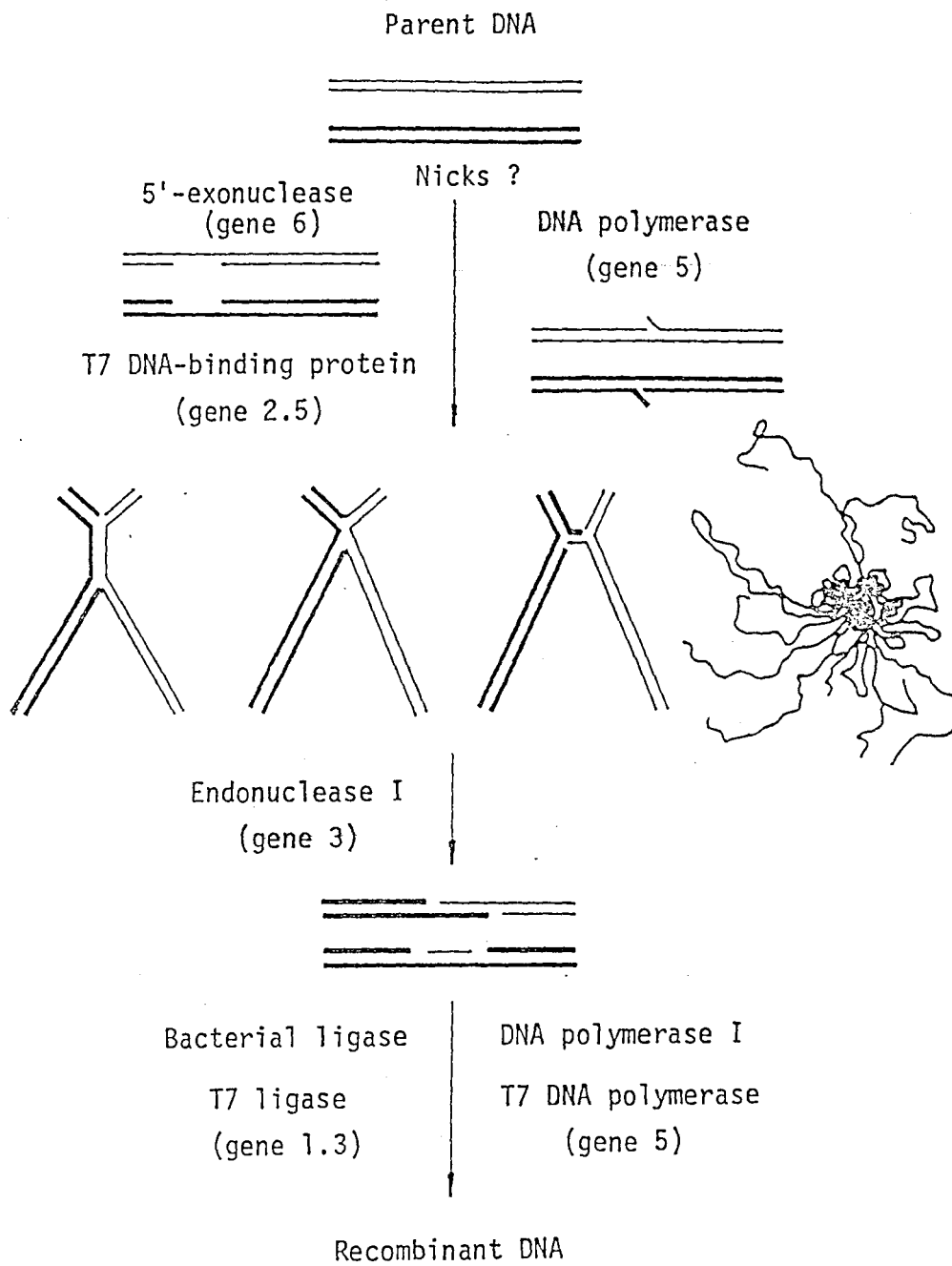
Figure I-2.   Schematic representation of a process of genetic recombination in bacteriophage T7.

of double-stranded DNA (Alberts and Frey, 1970; Sigal et al., 1972; Scherzinger

et al., 1973) and participate in replication (Epstein et al., 1963;

Meyer et al., 1979; Chapter III), recombination (Tomizawa et al., 1966;

Glassberg et al., 1979; Chapter III) and repair (Bernstein, 1981; Glassberg

et al., 1979; Johnson, 1977; Chapter III).   Their functions have not

been well known except they preferentially bind to single-stranded DNA.

They have a similar structure; carboxyl terminal region is composed

of many acidic amino acids (Williams et al., 1980; Sancar et al., 1981;

Dunn and Studier, 1981).   The carboxyl terminal region of T4 gene 32

protein and E. coli DNA-binding protein plays a regulatory role of its

function (Moise and Hosoda, 1976; Williams et al., 1981).   And I show

in this paper that the carboxyl terminal region of T7 DNA-binding protein

also plays a similar role (Chapter IV).   They interact with replication

enzymes and recombination enzymes (Mosig et al., 1978; Molineux and

Gefter, 1974, 1975).   Research about molecular mechanism of the

participation of single-stranded DNA-binding protein in DNA metabolism

has just started.

Before engaged in the study described in this paper, I was characteri-

zing in vitro recombination system which was prepared from T7-infected

cells (Ogawa et al., 1978).   The system mimicked in vivo system since

the formation of intermediate molecules in genetic recombination depended

on T7 exonuclease in both systems and the structure of intermediate

molecules formed in in vitro system was the same as that observed in in

vivo system.   Moreover, in addition to linear T7 DNA molecules,

circular plasmid DNA were also successfully used as substrates for the

formation of intermediate molecules.   This fact suggested that other

factors in addition to T7 exonuclease were involved in the formation of

intermediate molecules and prompted me to isolate other factor(s)

participating in genetic recombination.   So, I developed a new simple

method, DNA-cellulose method, for detecting the intermediate DNA molecules

easily, and found one of factors, a T7 DNA-binding protein (Chapter II).

Next, as a mutant defective in T7 DNA-binding protein had not been

isolated yet, I tried to isolate this T7 mutant using E. coli mutant

strain defective in DNA-binding protein to see the character of T7 mutant

in DNA-binding protein (Chapter III).   The isolated mutant revealed that

T7 DNA-binding protein participates in genetic recombination as well as

DNA synthesis and repair.   Lastly, I purified a mutant DNA-binding

protein coded by the isolated mutant and characterized its properties by

comparing with those of wild-type protein.   These analyses revealed

that mutant protein seems to have a defect in a regulatory portion of

its function.   From the results described in this paper, the participation

of T7 DNA-binding protein in DNA metabolism and the functions of it

has been cleared.

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II

THE PARTICIPATION OF T7 DNA-BINDING PROTEIN IN

IN VITRO T7 GENETIC RECOMBINATION

ABSTRACT

Recombination reactions were performed between ColE1 DNA bound to cellulose (DNA-cellulose) and ^{3}H-labelled ColE1 DNA in a crude extract of T7-infected cells. The amount of binding of radioactivity to DNA-cellulose depended on the presence of T7 exonuclease which is indispensable for genetic recombination to occur, and the binding reaction was specific for homologous DNA. Applying this method to the purification of enzymes which are essential for T7 genetic recombination, a protein factor was found in the T7-infected cells, which work in cooperation with T7 exonuclease. The protein was tentatively identified as the T7 DNA-binding protein, on the basis of purification and its molecular weight (32,000 daltons).

INTRODUCTION

The isolation and characterization of T7 phage recombination intermediates from cells infected with ^{32}P and BrdU labelled phages has been described (Tsujimoto & Ogawa, 1977). The recombination intermediates consisted of doubly branched molecules with X- or H-like configuration. The formation of these intermediates was shown to depend on the function of T7 gene 6, 5'-exonuclease. Transfection assay of these molecules revealed that they were infective, and that about 65% of them produced recombinant phages (Tsujimoto & Ogawa, 1978).

An identical type of branched molecules as observed above formed in the T7 recombination-packaging system developed by Sadowski and Vetter (1976), and here, too, the T7 gene 6 product was also indispensable in the formation of the branched molecules in vitro (Ogawa et al., 1978). Moreover, in this in vitro system, two molecules of circular plasmid DNA can form a figure-8 like structure with a long-pairing region in the presence of the 5'-exonuclease. This suggests that in the winding process for mutually complementary single-stranded regions created by the exonuclease, some stimulation factor(s) must participate in the extension of the pairing region.

In this paper, a new simple method will be described for the detection of fused molecules between two plasmid DNAs, and will show that at least one causal factor is the T7 DNA-binding protein.

MATERIALS AND METHODS

Materials and methods were those described in previous research (Tsujimoto & Ogawa, 1977, 1978; Ogawa et al., 1978) with the exception of the following.

Preparation of DNA-agarose

DNA-agarose was prepared by embedding alkali-denatured calf-thymus DNA, type I (Sigma), in 2% agarose (Sigma) according to the method of Shaller et al. (1972). This DNA-agarose contained 1.5 mg DNA/bed volume (ml) determined by the amounts of nucleic acids freed after treatment with DNase I (50 µg/ml) in 0.1 M Tris-HCl (pH 7.4), 10 mM MgSO$_4$ at 37°C for 1 hr.

Preparation of DNA-cellulose

DNA-cellulose was prepared by Litman's method (1968). Open circular ColE1 DNA (2-3 mg/ml) or calf-thymus DNA type I (Sigma) (2-3 mg/ml), was used for binding DNA to cellulose (Whatman CF-11). About fifty percent of the DNA was bound using this method. The amount of DNA bound to cellulose were determined by the same method as used for the preparation of DNA-agarose.

Preparation of open circular ColE1 DNA

Cleared lysate (50-100 ml) was prepared from A745(ColE1 thy$^-$) cells (Sakakibara & Tomizawa, 1974) following the method of Clewell and Helinski (1969). The lysate was heated at 70°C for 10 min and denatured protein was removed by centrifugation. Two volumes of cold

ethanol were added and the precipitate was collected by centrifugation. The pellet was dissolved in 2–5 ml of 20 mM Tris–HCl (pH 7.4) containing 5 mM EDTA, and treated with RNase A (50 µg/ml) at 37°C for 1 hr. The residual protein was removed by phenol extraction. The phenol was removed by ether and the solution was applied to Sephadex G–200 (3.2 cm x 17 cm) equilibrated with 20 mM Tris–HCl (pH 7.4) containing 5 mM EDTA. The DNA appearing in void volume was precipitated with ethanol and redissolved in 2–5 ml of 0.1 M Tris–HCl (pH 7.4) containing $MgSO_4$.

For converting covalently closed circular form of ColE1 DNA to open circular form, the DNA solution (2–3 mg/ml) was treated with 2×10^{-3} µg/ml DNase I in 0.1 M Tris–HCl (pH 7.4) containing 10 mM $MgSO_4$ at 30°C for 10–30 min. The reaction was stopped by the addition of 20 mM EDTA and the completed conversion was tested by agarose electrophoresis. The DNase was removed by phenol extraction, and dialyzed against 10 mM Tris–HCl (pH 7.4) containing 1 mM EDTA. Open circular ColE1 DNA was used for the preparation of DNA–cellulose.

Open circular ^{3}H–labelled ColE1 DNA was also obtained by this method after the DNA had been isolated by ethidium bromide–CsCl equilibrium density gradient centrifugation (Ogawa et al., 1978).

Preparation of T7 5'–exonuclease

T7 5'–exonuclease coded by gene 6 was purified by Shinozaki and Okazaki method (1979) except that here 594endA strain was used and cells were sonicated. The T7 5'–exonuclease used in the following experiments was phosphocellulose eluate. A unit of enzyme activity is defined as the amounts of enzyme producing 1 nmol of acid soluble nucleotides for 15 min at 37°C.

Preparation of the extract of T7-infected and uninfected cells

The cell suspension of infected cells in T7 diluent was prepared as in previous research (Ogawa et al., 1978), as was the suspension of uninfected cells.   Cells were disrupted by sonication (Branson Sonifier cell disrupter 185) and cell debris were spun down at 20,000 x g for 10 min.   The resultant supernatant is referred to the extract.

Fractionation of the extract of T7-infected and uninfected cells

T7 2am 3am 4am 5am 6am phage (Tsujimoto & Ogawa, 1977) was added at a multiplicity of 10 to the culture of 594endA (1.5 l) grown to 10^9/ ml at 37^oC in L-broth.   After incubation at 37^oC for 15 min, the infected cells were harvested by centrifugation at 0^oC.   The cells were suspended·in 20 ml of 20 mM Tris-HCl (pH 7.4) containing 1 mM EDTA, 1 mM 2-mercaptoethanol and 0.1 M NaCl, and disrupted by sonication in an ice water bath.   After removing cell debris, the supernatant (29 ml) was added by a one-tenth volume of 20% (W/V) streptomycin sulfate and stirred for 30 min at 0^oC, and then centrifuged at 15,000 x g for 40 min.   The protein in the supernatant (29 ml) was precipitated with the addition of ammonium sulfate (0.45 g/ml) and 1 N NaOH (0.05 ml/10 g $(NH_4)_2SO_4$), and the resulting precipitate was collected by centrifugation at 15,000 x g for 20 min.   The protein pellet was dissolved in 10 ml of buffer A (20 mM Tris-HCl (pH 7.4), 5 mM EDTA, 1 mM 2-mercaptoethanol, 10% glycerol) containing 0.3 M KCl and dialyzed against 500 ml of the same buffer overnight.   To remove residual nucleic acids, the dialyzed fraction (total 12 ml, 33 mg protein/ml) was applied to a DEAE-cellulose (Brown) column (3.2 cm^2x 12 cm) previously equilibrated with buffer A containing 0.3 M KCl .   The pass through fractions were pooled (50 ml), and precipitated with ammonium sulfate as above.   The pellet was

suspended in 3 ml of buffer A containing 0.4 M KCl and dialyzed

overnight against 300 ml of the same buffer.   A two milliliter

sample of the dialyzed DEAE fraction (45 mg/ml, A_{280}/A_{260}=1) was diluted

by half with buffer A and applied to a single-stranded DNA-agarose

column (0.78 cm^2 x 3.2 cm) equilibrated with buffer A containing 0.2 M

KCl.   The bound protein was eluted in the buffer with five column volumes

having a stepwise gradient, increasing in KCl concentration-0.2, 0.6,

1.0 and 2.0 M.

Polyacrylamide gel electrophoresis

    Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

was performed in 7.5% gels following Shapiro et al. (1967).
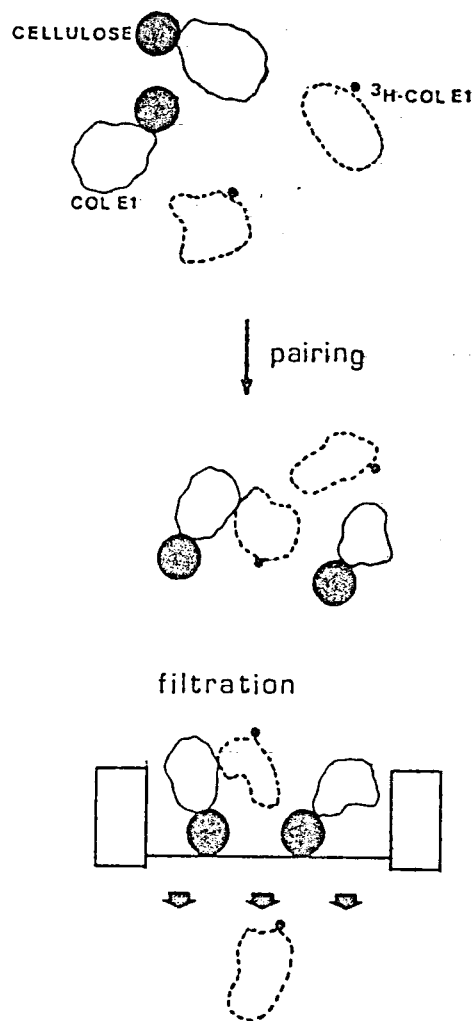
DNA-CELLULOSE ASSAY

Figure II-1.   A schematic representation of DNA-cellulose assay

See RESULTS for details.

A new and simple method was used to detect the formation of the
fused molecules between two plasmid DNAs.   Figure II-1 shows the
principle behind this method.   Open circular ColE1 DNA was bound to
cellulose by Litman's method, and the DNA-cellulose was incubated with
the mixture of ^{3}H-labelled ColE1 DNA and either the T7-infected cell
extract or partially purified enzymes.   After incubation, the reaction
mixture was filtered through filter paper.   When fusion occurs between
ColE1 DNA bound to cellulose and ^{3}H-labelled ColE1 DNA, ^{3}H-radioactivity
is retained on the filter paper together with ColE1 DNA-cellulose.

Using this assay system, a time course experiment to fuse molecules
was carried out in T7-infected cell extract.   An aliquot of ColE1
DNA-cellulose powder (containing about 20 μg DNA) was added to 50 μl of
the extract of T7 2am 3am 4am 5am 6$^+$ infected cells containing 2 μg of
open circular ^{3}H-labelled ColE1 DNA (2 x 10^5 cpm).   After incubation
for increasing time periods at 30°C, the reaction was terminated by
the addition of 5 ml of 10 mM Tris-HCl (pH 7.4) containing 5 mM EDTA
and 0.1% SDS, then filtered, and washed with 35 ml of the same buffer.
The radioactivity retained on filter paper increased with incubation
time until 2 hr and then levelled off (Fig. II-2).   At this levelling
point, the radioactive fraction retained was about 0.4% of the input.
When calf-thymus DNA-cellulose was used instead of ColE1 DNA-cellulose,
the radioactivity retained after 6 hr was less than 0.02% of the input.
This activity was almost the same radioactivity retained that the reaction
was omitted.

When an extract lacking T7 exonuclease was used, the amount of   .
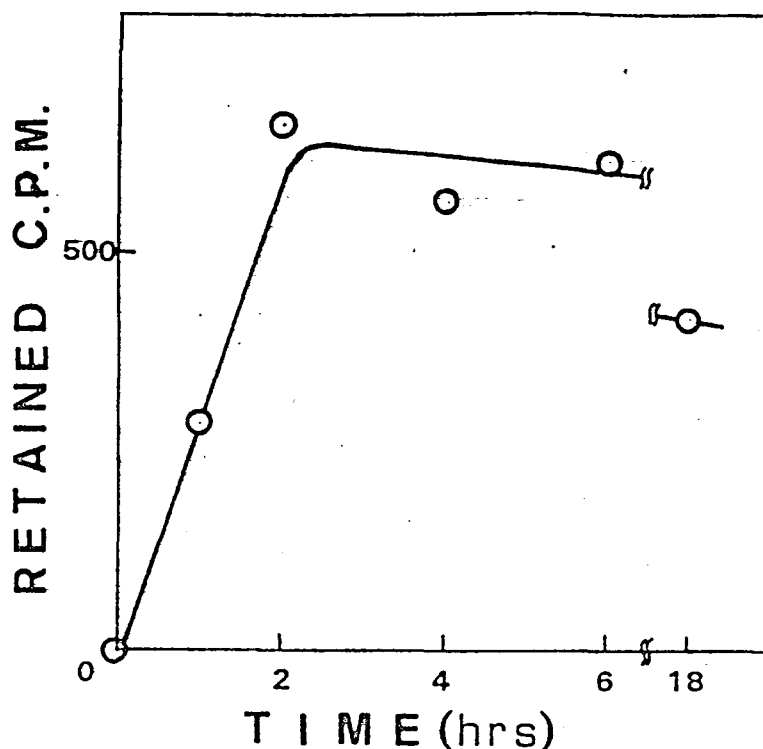
Figure II-2.   A time course experiment on the radioactivity retained by

DNA-cellulose in the extract.   An aliquot of ColE1 DNA-cellulose powder

(containing about 20 μg DNA) was added to 50 μl of a mixture composed of

T7 2am 3am 4am 5am 6^+ - infected cells and 2 μg of ^{3}H-labelled ColE1 DNA

$(2 \times 10^5$ cpm).   After incubation for various times at 30°C, the 5 ml of

10 mM Tris-HCl (pH 7.4) containing 5 mM EDTA and 0.1% SDS was added to

the mixture, then filtrated and washed with 35 ml of the same buffer.

The radioactivity retained on a filter was counted using a scintillation

counter.

Table II-1

Fractionation of binding activity

Fraction	Infected		Uninfected	
	Specific	Total	Specific	Total
Sonication	1(unit/mg)	600(units)	0	0
DEAE-fraction	120	16,200	0	0
DNA-agarose				
0.2 M KCl	47	6,000	40	5,000
0.6 M KCl	0	0	0	0
1.0 M KCl	1,100	670	0	0
2.0 M KCl	0	0	0	0

Fractionation procedures follow those described in MATERIALS AND
METHODS. An aliquot of each fraction was added to the reaction mixture
which contained T7 5'-exonuclease (0.1 unit), ^{3}H-labelled nicked open
circular ColE1 DNA (1 µg), ColE1 DNA-cellulose (20 µg DNA), 10 mM Tris-
HCl (pH 7.4), 0.1 M NaCl, 10 mM $MgSO_4$ and 1 mM 2-mercaptoethanol, and
followed incubation at 30°C for 2 hr.   Radioactivity bound to ColE1 DNA-
cellulose was measured as retained on a filter.   One unit of activity
is defined as; 1 unit = 1 ng of the DNA bound to ColE1 DNA-cellulose per
1 milliunit T7 Exonuclease after 2 hr incubation at 30°C.   The amounts
of the DNA bound to ColE1 DNA-cellulose were calculated from radioactivity
T; T = A - (B + C).   A: Radioactivity retained after incubation of the
reaction mixture containing each fraction (0.4 - 2 % of the taotal input
was reatained).   B: Radioactivity retained after incubation of the
reaction mixture without T7 exonuclease and with each fraction (0.4 -
0.6 % of the total input was reatained).   C: Radioactivity retained
after incubation of the reaction mixture alone (0.4 - 0.7% was retained).

radioactivity retained was one-sixth of that when T7 exonuclease was

present.   The addition of purified T7 exonuclease (0.18 unit) to an

extract lacking exonuclease more than doubled the radioactivity.

When an extract of T7-uninfected cells was used, all radioactivity was

lost even if T7 exonuclease was added.   Thus protein factor(s) coded

by the T7 genome participate in fusion between the two plasmid DNAs after

the addition of T7 exonuclease to the extract.

Fractionation of the extract of T7-infected and uninfected cells

    The cell extract was prepared from T7 exonuclease-minus phage

(T7 2am 3am 4am 5am 6am) infected cells, and DNA was removed by

precipitation with streptomycin sulfate and DEAE-cellulose column

chromatography.   Then the DEAE through fraction was applied to a

single-stranded DNA-agaraose column equilibrated with 0.2 M KCl.   The

bound protein was eluted from the column by stepwise increase of salt.

An aliquot of each fraction was added to the assay mixture which contained

T7 exonuclease, ^{3}H-labelled nicked open circular ColE1 DNA and ColE1 DNA-

cellulose.   The relative amounts of the radioactivity bound per mg

protein added to the reaction mixture are shown in Table II-1.

Results with uninfected cells are also shown for comparison.   The

activity facilitating ^{3}H-DNA association with DNA-cellulose was found

in infected cells, and appeared in 0.2 M and 1 M KCl eluates of DNA-

agarose chromatography.   In uninfected cells such activity only appeared

in the 0.2 M eluate.   Therefore, it was concluded that activity in

the 1 M eluate was derived from the protein coded by the T7 genome.

Identification of the factor coded by T7 phage

    The protein in the 1 M eluate was analyzed by SDS polyacrylamide
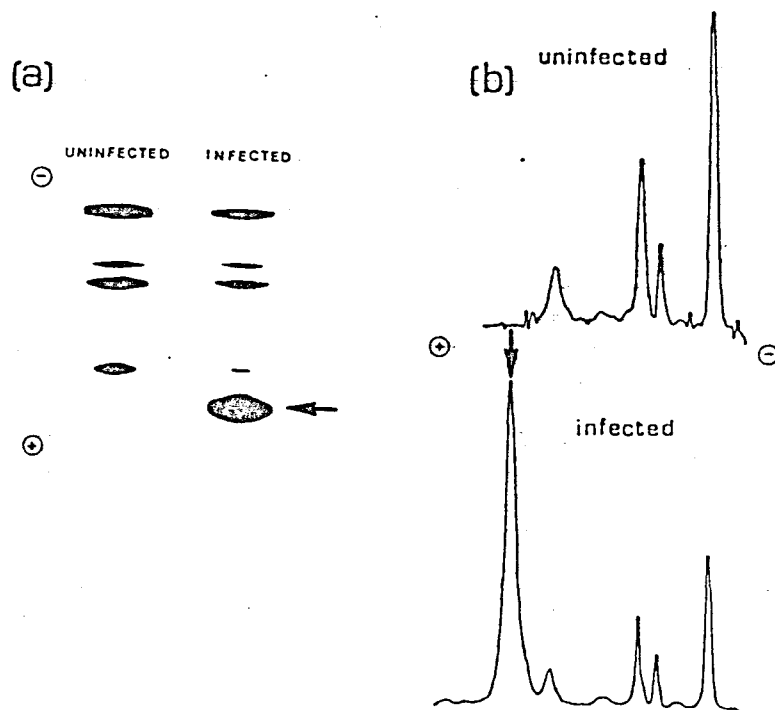
Figure II-3.   Profiles of protein bands of 1 M eluate from DNA-agarose

column on SDS-polyacrylamide gels and their scanned profiles.

(a) A photograph of protein bands stained on SDS-polyacrylamide gels.

Left side; proteins from uninfected cells (5 µg).   Right side; proteins

from infected cells (5 µg).   (b) The densitometry tracing of the

photograph.   A characteristic protein from infected cells is indicated

by an arrow.   The densitometry was carried out using a Toyo digital

densitorol DMU-33C.

gel electrophoresis (Weber & Osborn, 1969).   A characteristic protein

in the 1 M eluate of infected cells, which was not found in the same

eluate of uninfected cells, had a molecular weight of 32,000 daltons

and a purity greater than 63% (Fig. II-3, II-4).   From its molecular

weight and its ability to bind with DNA-agarose, it is thought to be

T7 DNA-binding protein reported by Reuben and Gefter (1973).   On the

other hand, although the 0.2 M eluate seems to contain the proteins

coded by the host genome, further purification is required.   These

results imply that T7 exonuclease cooperating with the T7 DNA-binding

protein is capable of forming fused molecules.
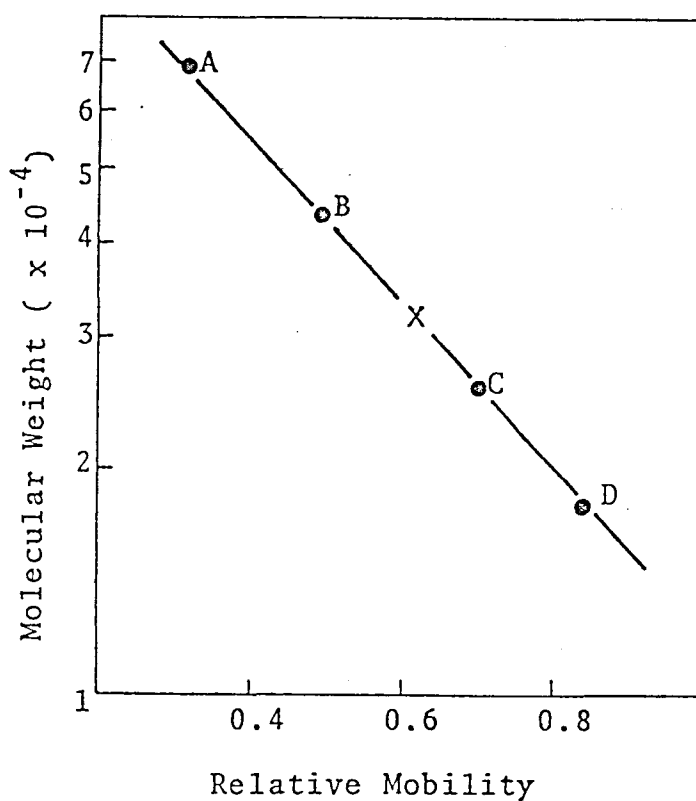
Figure II-4.   Determination of molecular weight of characteristic

purified protein extracted from infected cells by SDS-polyacrylamide

gel electrophoresis.   The method followed was generally as described

in Weber and Osborn (1969).   Protein standards were A: albumin (68,000

daltons) B; ovalbumin (43,000 daltons)   C; chymotrypsinogen A (25,700

daltons)   D; myogloblin (17,800 daltons).   Mobilities are expressed

relative to the marker dye, bromphenol blue.

Mark(X) indicates the position of the characteristic protein in infected

cells.

DISCUSSION

In genetic recombinaiton of T7 phage, the gene 6 protein,
exonuclease was assumed to have a primary role in fusion of two DNAs
(Tsujimoto & Ogawa, 1977).   However, this protein alone seemed unable
to catalyze the formation of branched molecules (Ogawa et al., 1978).
Therefore, the factors involved in recombination acting together with
T7 exonuclease were explored, using the new and simple method described
here.   One protein factor was identified as the T7 DNA-binding protein
from both its molecular weight and its binding characteristics during
DNA-agarose column chromatography.   The role of T7 DNA-binding protein
in genetic recombinaiton will be shown in Chapter. IV.   In bacteriophage
T4, it has been reported that products of gene 32 (DNA-binding protein)
and products of gene 46 and gene 47 (exonuclease) are required for
the formation of the intermediate molecules of genetic recombination in
infected cells (Tomizawa et al., 1966; Hosoda, 1976).   These facts
imply that the T7 DNA-binding protein is necessary for genetic recombination
in T7-infected cells.

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III

T7 PHAGE MUTANT DEFECTIVE IN DNA-BINDING PROTEIN

III-A

THE ISOLATION AND CHARACTERIZATION OF T7UP-2 PHAGE

WHICH IS DEFECTIVE IN T7 DNA-BINDING PROTEIN

ABSTRACT

A T7 phage mutant, UP-2, in the gene for T7 DNA-binding protein

was isolated from mutants which could not grow on 594ssb-1 bacteria

but could grow on C600ssb-1 and 594 bacteria.   The mutant phage

synthesized a smaller polypeptide (28,000 daltons) than T7 wild-type

DNA-binding protein (32,000 daltons).   DNA synthesis of the UP-2

mutant in 594ssb-1 cells was severely inhibited and the first round

replication was found to be repressed.   The abilities for genetic

recombination and DNA repair were also low even in permissive hosts

compared with those of wild-type phage.   Moreover, recombination

intermediate T7 DNA molecules were not formed in UP-2 infected non-

permissive cells.   The gene that codes for DNA-binding protein is

referred to as gene 2.5 since the mutation was mapped between gene 2

and gene 3.

INTRODUCTION

T7 DNA-binding protein stimulates T7 DNA synthesis in vitro
(Reuben & Gefter, 1973; Scherzinger et al., 1973; Scherzinger &
Klotz, 1975; Richardson et al., 1978), and also participates in in
vitro recombination in cooperation with T7 exonuclease (Araki & Ogawa,
1981; Chapter II). These results suggest that T7 DNA-binding protein
is involved in T7 DNA replication and recombination. However, this
assumption remains unproven. since mutants defective in the gene for
DNA-binding protein have not been isolated. The isolation of such
mutants was considered impossible due to the anticipated complementation
of such a defect by host bacterial DNA-binding protein, since E. coli
and T7 DNA-binding proetins were known to be mutually interchangeable
in in vitro DNA replication (Reuben & Gefter, 1974; Scherzinger &
Klotz, 1975). Recently, one dna mutant of E. coli was found to be
defective in the activity of DNA-binding protein (Meyer et al., 1979).
Utilizing this mutant, I isolated a T7 phage mutant defective in DNA-
binding protein . The genetic and biochemical analyses of this mutant
are presented in this paper.

MATERIALS AND METHODS

Bacteria and phages

Bacterial strains used in this study are listed in Table III-1.

Bacteriophage T7 except the mutants isolated in this work was supplied

from Dr. Studier.

Media and Buffers

M9 medium (Clowes & Hayes, 1968) was used for labelling of T7-

directed proteins with ^{35}S-methionine.   Modifies M9 medium containing

13 mM Na_2HPO_4, 7 mM KH_2PO_4, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.05% NaCl, 0.1%

NH_4Cl, 0.001% gelatin, 0.2% glucose and 0.5% casamino acids was used

for measurement of T7 DNA synthesis, Cas-λ broth (Tsujimoto & Ogawa,

1977) for density labelling experiment of T7 DNA replication.   T-broth

(Tsujimoto & Ogawa, 1977) was used for phage crosses, L-broth (Ikeda &

Tomizawa, 1965) for preparation of T7 phage, and T-agar containing T-

broth and 1.0% agar for titration of T7 phage.   T7 buffer containing 10

mM Tris-HCl (pH 7.4), 1 mM $MgSO_4$, 0.01% gelatin and 5% NaCl was used

for dilution of T7 phage and for ultraviolet light (UV) irradiation of

T7 phage.   SSC contains 0.15 M NaCl and 0.015 M sodium citrate (pH 7.0).

SSC diluted a half with H_2O is referred to as 1/2 SSC.

Isolation of T7 mutant phages

The procedure of Studier (1969) was slightly modified.   To a log-

phase culture (2 x 10^8/ml) of Q1 cells, N-methyl-N'-nitro-N-

nitrosoguanidine was added to 40 µg/ml, followed by addition of T7

phage at a multiplicity of infection of 0.1.   After shaking at 37°C

Table III-1

E. coli K12 strains used in this work.

Strain	Relevant properties	Source or Reference
594	sup^+	Campbell (1965)
594(pDR1996)	$sup^+ tet^r amp^r$	transformation with pDR1996 obtained from Dr. W. D. Rupp
WD00	sup^+ thyA deo malB A derivative of 594	Ogawa (1975)
594lexC113	sup^+ thyA deo	PAM2611 X WD00, selection for $malB^+$ lexC113
594ssb-1	sup^+ thyA deo	P1(SG1635)→WD00, selection for $malB^+$ ssb-1
594metEmalB	sup^+ thyA deo	spontaneous metE mutant of WD00
594trxAmalB	sup^+ thyA deo	P1(JM110)→594metEmalB, selection for $metE^+$ trxA
594trxAssb-1	sup^+ thyA deo	P1(SG1635)→594trxAmalB selection for $malB^+$ ssb-1
Q1	glnU	obtained from Dr. E. Signer
Q1(pDR1996)	$glnU\ tet^r amp^r$	transformation with pDR1996 obtained from Dr. W. D. Rupp
C600	glnU thyA deo thr leu	Ogawa and Tomizawa (1967)
C600malE	glnU thyA deo thr leu	obtained from Dr. Epstein
C600lexC113	glnU thyA deo	PAM2611 X C600, selection for $thr^+ leu^+$ lexC113
C600ssb-1	glnU thyA deo thr leu	P1(SG1635)→C600malE, selection for $malE^+$ ssb-1
JC1557	sup59	Clark et al. (1966)
JC1557uvrA	sup59	obtained from Dr. Y. Yamamoto.
PAM2611	Hfr, lexC113	obtained from Dr. B. F. Johnson
SG1635	glnU thyA ssb-1	Sevastopoulos et al. (1977)
JM110	trxA thyA	Mark et al. (1977)

for 2 hr, a few drops of chloroform and 1/5 volume of 25% NaCl solution were added. The lysate was diluted and plated on C600ssb-1 at approximately 100 plaques per plate, and then the plates were overlayered with 594ssb-1 bacteria. After incubation at 37°C for 4-5 hr turbid plaques were picked and mutant phages which could make plaques on C600ssb-1 and 594 but not on 594ssb-1 were selected.

Analysis of T7-directed proteins

Cells of 594ssb-1 grown to 4-5 x 10^8/ml in M9 medium at 30°C were irradiated by UV light (600 J/m^2), shaken for 30°C for 30 min, and then infected with T7 phage at a multiplicity of 10. After 5 min incubation at 30°C, 10 µCi of ^{35}S-methionine (1,000 Ci/mmol) was added to 1 ml of the culture and incubation was continued for 15 min. The culture was chilled, centrifuged and the pellet was resuspended in 0.1 ml of 62.5 mM Tris-HCl (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue. After heating for 4 min in a boiling water bath, the sample (10 µl, about 200,000 cpm) was subjected to SDS-polyacrylamide gel electrophoresis (Studier, 1973) using a slab gel of 12.5% polyacrylamide, and run 14 cm with a marker dye (bromphenol blue). The gel was stained with coomassie brilliant blue G-250 to determine the position of purified T7 DNA-binding protein added as a marker, and then dried and examined autoradiographically using Kodak XR-1 X-ray film. T7 DNA-binding protein was purified by the method described previously (Araki & Ogawa, 1981; Chapter II).

Measurement of T7 DNA synthesis

Cells were grown in modified M9 medium containing 6 µg thymidine/ml to 2 x 10^8/ml and irradiated with UV at a dose of 300 J/m^2 for 594ssb-1,

C600ssb-1, WD00, or 1,000 J/m^2 for C600.   After incubation at 37°C for

30 min, 1 μCi ^{3}H-thymidine (52 Ci/mmol) /ml was added to the culture.

After 5 min, cells were infected with phage at a multiplicity of 10.

At 10 min intervals, 0.5 ml of the culture was taken into 0.5 ml of

chilled 10% trichloroacetic acid containing 100 μg unlabeled thymidine/ml.

The samples were filtered with a glass filter, washed with 5% trichloroacetic

acid, and radioactivity retained on the filter was measured in a liquid

scintillation counter.

Pulse labelling

    Thymine requiring cells were grown in modified M9 medium containing

6 μg thymidine/ml to 2 x 10^8/ml and infected with phage at a multiplicity

of 10.   Then at 3 min intervals, 0.5 ml of samples was mixed with 10 μl

(1 μCi) of ^{3}H-thymidine (52 Ci/mmol) in a tube and placed at 37°C for

1 min.   Incorporation was terminated by adding 0.5 ml of chilled 10%

trichloroacetic acid containing 100 μg unlabelled thymidine/ml.

Radioactivity incorporated into the acid-insoluble fraction was

measured as described above.

Preparation of labelled phages

    ^{32}P-, ^{3}H- and BrdU(5-bromodeoxyuridine)-labelled phages were prepared

by the method of Tsujimoto and Ogawa (1977).

Density labelling experiments for T7 DNA replication

    Bacteria of 594ssb-1 were grown to a density of 1 x 10^8 cells/ml

in Cas-λ broth containing 6 μg thymine/ml at 30°C, harvested by

centrifugation, resuspended in Cas-λ broth containing 10 μg BrdU and

1 μg thymine/ml, and incubated further at 30°C.   When the cell concentration

reached 2×10^8/ml, the incubation-temperature was shifted to 37°C and the culture was further incubated for 30 min.   After addition of ^{32}P-labelled T7 phage at a multiplicity of 10, the infected cells were incubated for an additional 15 min, harvested by centrifugation and suspended in SSC containing 10 mM EDTA and 500 μg lysozyme/ml.   The cells were lysed by 3 cycles of freezing and thawing, and mixed with 3 times volume of 1/2 SSC containing N-lauroyl sarcosinate (final concentration 1%) and Pronase (final concentration 1 mg/ml, self digested at 37°C for 4 hr and heated at 80°C for 3 min).   After incubation of the mixture at 37°C for 60 min, CsCl was added to a final density of 1.72 g/cm^3 and the sample of 5 ml was centrifuged at 36,000 revs/min for 40 hr at 15°C in a Spinco 40 rotor.   Each sample contained less than 1×10^9 infected cells.

Isolation of intermediate T7 DNA genetic recombinant molecules

Bacteria of 594trxAssb-1   were grown to 2×10^8 cells/ml in T-broth at 30°C.   After shaking at 37°C for 30 min to inactivate E. coli DNA-binding protein, the cells were infected with ^{32}P- and BrdU-labelled T7 phage each at mutiplicity of 20, and incubated for an additional 15 min. The infected cells were harvested and resuspended in SSC containing 10 mM EDTA.   Extraction of DNA and centrifugaiton of DNA in a CsCl solution were carried out as those for density-labelling experiments. The half-heavy density fraction in the CsCl gradient was recentrifuged in the presence of 0.015% sodium N-lauroyl sarcosinate.   Peak fractions at the half-heavy density were dialyzed agianst SSC containing 2 mM EDTA at 0°C for 2 hr, diluted 2 fold with water and treated with 100 μg RNase A/ml.

Measurement of recombination frequency

lysates of two parental phage prepared freshly in the same day were

diluted with T-broth containing 2 mM $MgSO_4$ to a concentration of

4×10^9 phage/ml. The phage solutions of 0.25 ml each were mixed and

then 0.5 ml of fresh culture grown in T-broth to 2×10^8 cells/ml was

added. The mixture was kept standing at $37^{\circ}C$ for 5 min, and then

treated with phage T7 specific antiserum at a final K value of 3 for 5

min at $37^{\circ}C$. The infected cells were diluted $1 : 10^4$ with T-broth and

aliquot was plated with Q1 cells to measure infective centers. The

remainder was divided into two portions. One was incubated at $37^{\circ}C$

for 45 min to allow phage growth, and another was treated with $CHCl_3$ to

measure number of unadsorbed phage. The phage burst was determined

with indicator strain of Q1. The total number of recombinants was

obtained by doubling the number of plaques on 594 after correcting for

the plating efficiency relative to that on Q1.

UV inactivation of T7 phage

T7 phage was diluted with T7 buffer to a concentration of 1×10^9/ml

and irradiated with various UV doses. The dose rate was measured

by UV Radiometer C-254 (Toshiba). Irradiated T7 phage was plated with

various bacterial strains, and subsequent incubation was carried out

in the dark at $37^{\circ}C$.

RESULTS

Isolation of a T7 phage mutant defective in T7 DNA-binding protein

Bacterial DNA-binding protein seems to be able to replace T7 DNA-binding protein in T7 DNA replication in vitro (Reuben & Gefter, 1974; Scherzinger and Klotz, 1975). Therefore, for isolation of T7 phage mutant defective in T7 DNA-binding protein, bacteria carrying a ssb-1 mutation which produces a temperature-sensitive DNA-binding protein (Meyer et al., 1979) were used. From 50,000 plaques of mutagenized T7 phage, 14 mutants which could grow on 594 and C600ssb-1 but not on 594ssb-1 bacteria, were obtained. These mutants could be classified into 7 groups by complementation studies and the genes mutated in six groups of them were identified by complementation with known mutant T7 phage (Table III-2). A representative mutant of each group was further analyzed for T7-directed proteins in DM455 bacteria (Fig. III-1). One of the mutants examined could not synthesize a polypeptide corresponding to DNA-binding protein (32,000 daltons) in 594ssb-1, and instead, synthesized a slightly smaller protein of 28,000 daltons as shown in Fig. III-1 and Fig. III-2 lane 2. In C600ssb-1 bacteria, this mutant phage UP-2 synthesized a small amount of a polypeptide corresponding to DNA-binding protein in addition to the smaller molecular weight protein (Fig. III-2 lane 3). Table III-3 shows plating efficiencies of the UP-2 mutant and the wild-type phage on various bacterial strains carrying ssb-1 or lexC113 mutation, which produce temperature-sensitive DNA-binding protein (Sancar and Rupp, 1979; Glassberg et al., 1979). The plating efficiencies of T7 wild-type phage on the mutant bacteria were slightly low (1/2 - 1/4), compared with that on the wild-type cells.


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Table III-2

Complementation Groups

| Group	Mutation	Gene	Function
I	UP-1, UP-6[b], UP-13[a]	3.5	Lysozyme
II	UP-2[b]	2.5	DNA-binding protein
III	UP-3, UP-4[b], UP-5, UP-11, UP-13[a], UP-15	1	RNA polymerase
IV	UP-7[b]	12	Tail protein
V	UP-8,[b] UP-16	3	Endonuclease I
VI	UP-12[b]	16	Head protein
VII	UP-14[b]	14	Head protein

Complementation test was carried out by spotting the two lysates (about 1×10^8 phage/ml) at the same place on the lawn of 594ssb-1. The results were confirmed by testing phage yield by mixed infection. Bacteria 594ssb-1 were grown to 2×10^8/ml in L-broth and infected with both phages to be tested in a multiplicity of 10, each. If the phage yield obtained was higher than that of single infection of each phage, two mutants were classified to be in different complementation groups. Mutant phages used for the identification of the mutation were as follows: T7 am193(gene 1), am29(gene 3), lys13a(gene 3.5), am3(gene 12), am140(gene 14), am9(gene 16).

a. A double mutant.

b. A representative mutation of each complementation group.
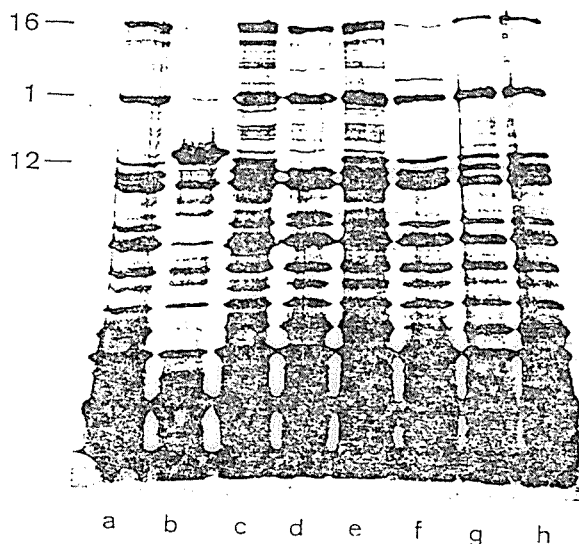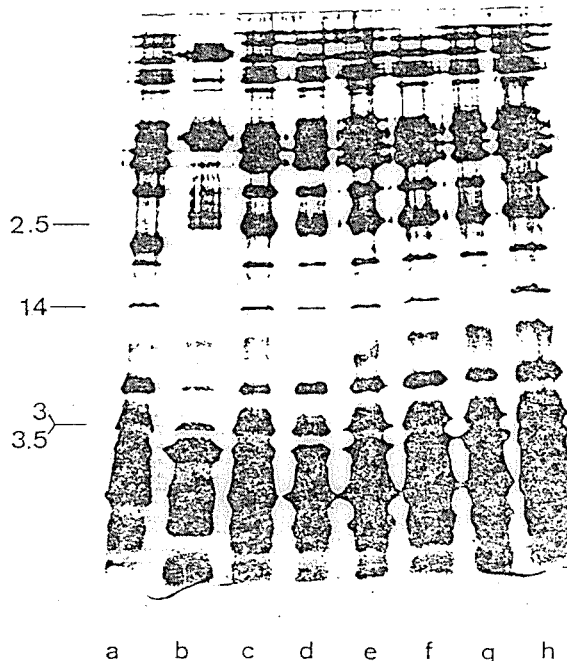
Figure III-1.   Profiles of the proteins synthesized by various T7
phages on SDS-polyacrylamide gel electrophoresis.

UV-irradiated (600 J/m^2) bacteria, DM455, were infected with various
T7 phages ((a) 2.5UP-2, (b) 1UP-4, (c) 3.5UP-6, (d) 12UP-7, (e) 3UP-8,
(f) 16UP-12, (g) 14UP-14, (h) wild-type), and proteins synthesized from
7 to 18 min after infection were labeled with ^{35}S-methionine at 37°C.
The cells were collected and the pellet was resuspended in 62.5 mM Tris-
HCl (pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and
0.001% bromophenol blue.   Then the suspension were subjected to electro-
phoresis on slabs of 7.5%(A) and 15%(B) polyacrylamide gel after denaturation
of proteins by heating.   The gel was dried and autoradiographed.   The
origin of electrophoresis is at the top in the figure.   The numbers
on the left side of photographs indicate the position of the proteins
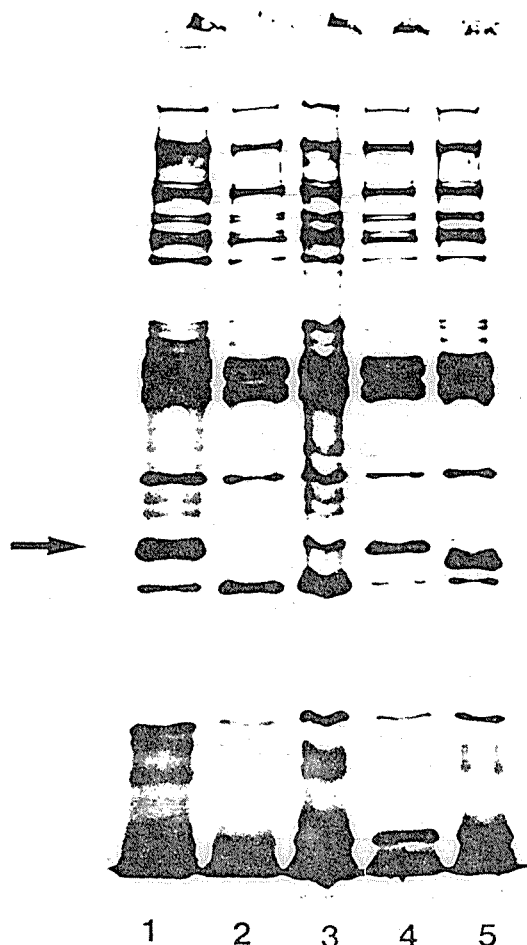directed by the corresponding T7 genes.

Figure III-2.   Profiles of the proteins synthesized by various T7 phages
on SDS-polyacrylamide gel electrophoresis.

UV-irradiated (600 J/m^2) 594ssb-1(lane1, 2, 3, 4, 5) or C600ssb-1
(lane 3) bacteria were infected with various T7 phages and were labelled
with ^{35}S-methionine from 5 to 20 min after infection at 37°C.   The
cultures were centrifuged and the pellet were resuspended in 62.5 mM
Tris-HCl(pH 6.8) containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol
and 0.001% bromophenol blue.   Then, the suspension were subjected to
12.5% SDS-polyacrylamide gel electrophoresis after denaturation of proteins
by heating.   The gel was stained, dried and autoradiographed.

The origin of electrophoresis is at the top in the figure.   The
arrow indicates the position of T7 DNA-binding protein determined by the
mobility of the purified DNA-binding protein run with the samples.   The
cells infected with UP-2 are lane 2, 3, the cells infected with wild-type
are lane 1, and the cells infected with r-2, r-3, revertants of UP-2,
are lane 4, 5, respectively.

The plating efficiencies of UP-2 phage on 594ssb-1 and 594lexC113 were very low. On the other hand, on 594 and Q1 strains, the plating efficiencies of the mutant were not significantly different from those of the wild-type phage. Thus the growth of the mutant UP-2 is completely dependent on the host SSB (DNA-binding protein) function in 594 strain.

From the T7 UP-2, some revertants which can grow on 594ssb-1 bacteria were isolated at 37°C at a frequency of about 10^{-6}. Their plating efficiencies with various bacterial strains are almost equal to those of the wild-type phage. The result with one representative revertant, T7r-2 was included in Table III-3. All revertants (3 strains) of UP-2 mutant synthesized a polypeptide corresponding to DNA-binding protein (two examples are shown in Fig. III-2 lane 4, 5). Therefore, with UP-2 mutant, inability to grow on ssb-1 or lexC113 mutant should be attributable to a defect in the DNA-binding protein. Slight difference with the mobility of the protein synthesized by r-3 phage in this method, was not observed if the analysis was carried out by a SDS-polyacrylamide gel electrophoresis described by Shapiro et al. (1967). This suggests that DNA-binding protein synthesized by r-3 has a normal size but a different isoelectric point caused by the insertion of different amino acid into the mutation site. However, even by this Shapiro's method the mobility of the protein synthesized by UP-2 was still different from the wild-type (data not shown). From the results described above, it is likely that the UP-2 mutation is occurred in the structual gene for T7 DNA-binding protein. And the UP-2 seems to be an amber mutation. But the UP-2 mutation is an opal mutant not an amber mutant by the nucleotide sequence of T7 UP-2 phage (See Chapter IV and Discussion).

Table III-3

Plating efficiency of T7 UP-2 mutant on various host bacteria

host strain	Phage strain		
	wild	UP-2	r-2
Q1	1.0	1.0	1.0
C600ssb-1	0.29	0.10	0.86
C600lexC113	0.54	0.28	0.93
594	0.75	0.46	1.1
594ssb-1	0.23	1.6×10^{-5}	0.86
594lexC113	0.49	1.6×10^{-3}	0.90

Plating efficiency is expressed as a relative number to that on Q1.

Mapping of the UP-2 mutation

Several crosses were carried out to map the UP-2 mutation.
The results of the two-factor crosses between the UP-2 mutant and the
am64(gene 2) or am29(gene 3) mutant are shown in Table III-4.   The
results suggest that the UP-2 mutation is located between gene 2 and
gene 3, nearer to gene 3.   A three-factor cross between the UP-2 mutant
and a double mutant, am64am29, confirmed the above conclusion (Table III-4,
the last line).

Based on these results, I propose the name of the gene that codes
for T7 DNA-binding protein as gene 2.5 according to the nomenclature of
the phage T7 genes originally proposed by Studier (1969).

Effect of the UP-2 mutation on phage DNA synthesis

The DNA synthesis of the mutant phage was examined by the incorporation
of ^{3}H-thymidine using UV-irradiated host cells.   Bacteria were irradiated
heavily with UV and infected with the wild-type phage or the mutant
phage in the presence of ^{3}H-thymidine.   At the each time after phage
infection, the radioactivity incorporated into the acid-insoluble fraction
was determined.   In C600 and C600ssb-1 bacteria, the mutant phage could
synthesize a slightly lower amount of DNA than the wild-type phage
(Fig. III-3).   In 594ssb-1 and WD00 bacteria, on the other hand,
DNA synthesis of the mutant phage was greatly suppressed.   However,
a distinction between two strains, 594ssb-1 and WD00 was observed
when pulse-labelling was carried out.   Results shown in Fig.III-4
were obtained by pulse-labelling with ^{3}H-thymidine for 1 min at 3 min
interval after phage infection.   In 594ssb-1 strain, the rate of
incorporation of thymidine by T7 UP-2 infection decreased rapidly by

Table III-4

Mapping of the UP-2 mutation

Cross	Recombination frequency(%)
am64 x am29	13.5
am64 x UP-2	13.0
am29 x UP-2	5.0
am64 am29 x UP-2	1.0

The procedures were described in MATERIALS AND METHODS. Bacterial strain, Q1, was used in these crosses. T7am64 phage is an amber mutant in gene 2 and T7am29 phage is that in gene 3.

The number of the recombinants was obtained by doubling the number of plaques on 594ssb-1 after correcting for plating efficiency of T7 wild-type relative to Q1, 0.23. And recombination frequency is expressed as a percentage of the recombinants among the total plaques on Q1.
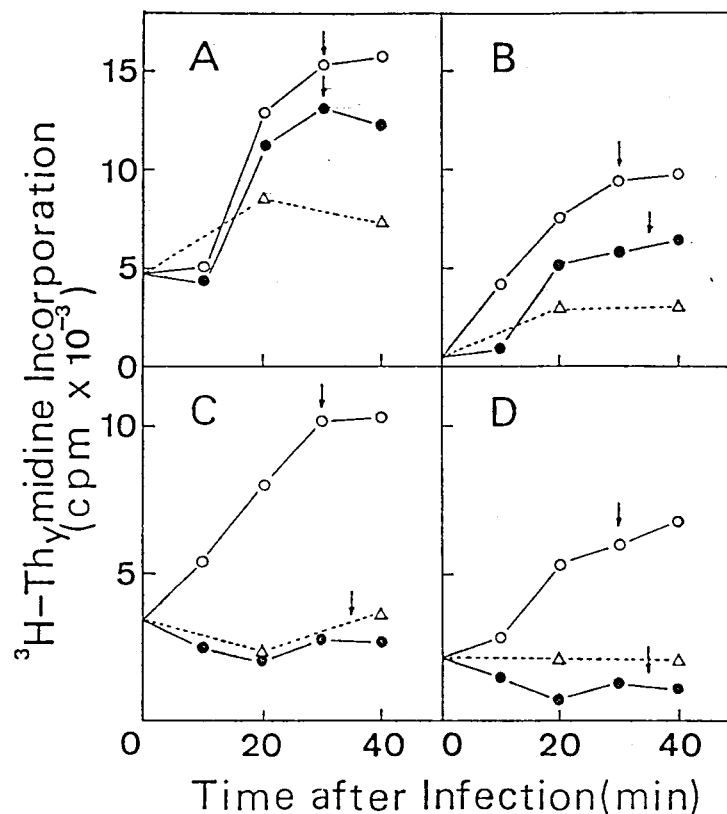
Figure III-3.   Time course of DNA synthesis in the T7 infected cells.

UV-irradiated various strains were infected with T7 wild-type or UP-2

phage and incubated in the presence of ^{3}H-thymidine at 37°C.   At 10 min

intervals, 0.5 ml of the culture was taken into 0.5 ml of 10% trichloro-

acetic acid containing 100 μg unlabeled thymidine/ml.   Radioactivity in

the acid-insoluble fraction was measured.   (A): C600,   (B): C600ssb-1,

(C): WD00,   (D): 594ssb-1,   (○): T7 wild-type,   (●): T7 UP-2,   (△): no

phage.   An arrow indicates the point at which the visible lysis occurred.
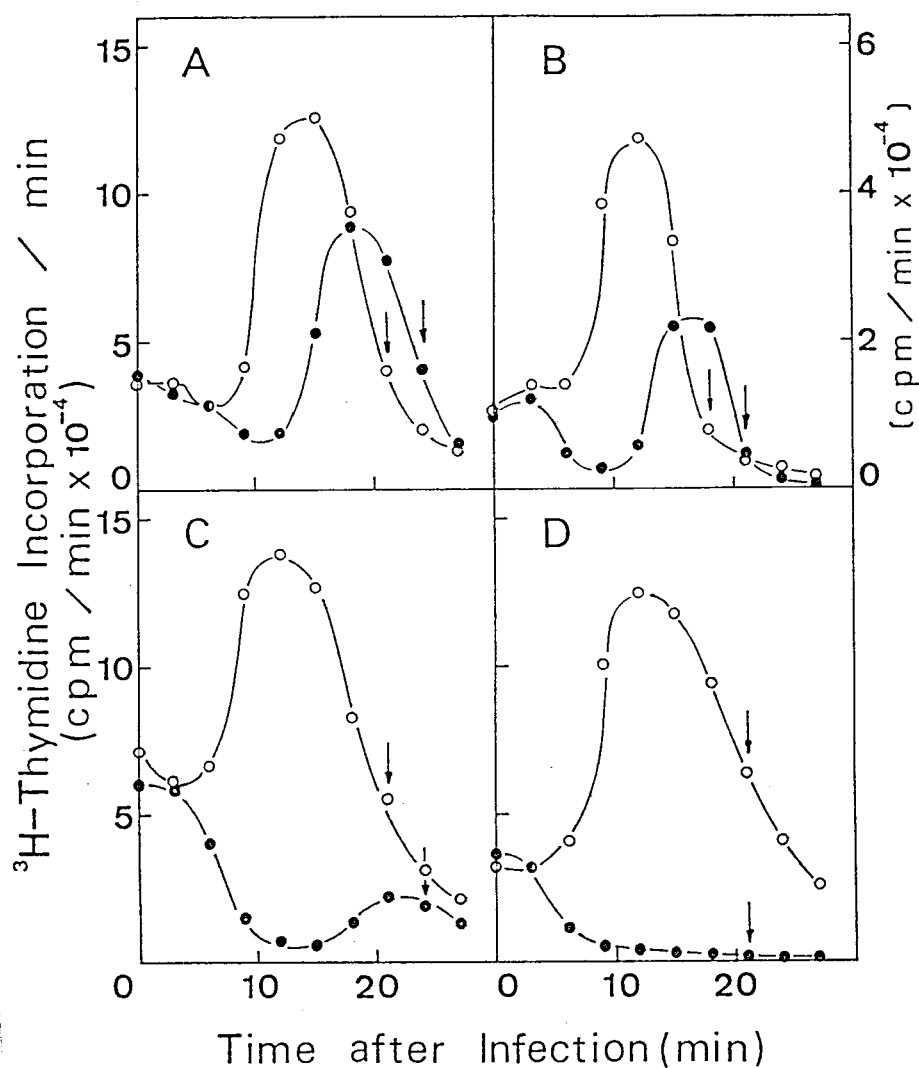
Figure III-4. Time course of the rate of the DNA synthesis after the infection of T7 phage.

Various cells, C600 (A), C600ssb-1 (B), WD00 (C) and 594ssb-1 (D), were infected with T7 wild-type (o) or UP-2 (●) at 37°C. A half milliliter of the culture was sampled at 3 min intervals and pulse-labelled for 1 min with ^{3}H-thymidine (1 μCi). Radioactivity incorporated in the acid-insoluble fraction was measured. An arrow indicates the point where the visible lysis occurred.

10 min and then decreased gradually with increasing time. This

decrease of the incorporation rate probably reflects the shutting

off of the host DNA synthesis and the absence of T7 DNA synthesis.

However, on WD00 strain, a significant amount of incorporation was

observed around 20 min after infection. This distinction probably

corresponds to the capability of plaque formation of the T7 UP-2 on

WD00 strain. In contrast, on both C600 and C600ssb-1 strains, a

maximal rate of the incorporation after the infection of the T7 UP-2

phage attained 60-70% of that of the wild-type phage, although its

attainment delayed for 5 min, compared with the cases of T7 wild-type

phage infection. With T7 wild-type phage infection, similar patterns

were obtained on all bacterial strains: a rapid increase of the rate of

DNA synthesis started around 7 min and the rate reached to maximum at

about 12 min, then decreased rapidly. These results show that the

complementation of a functional defect of the UP-2 mutation with the

bacterial function is not so effective as to restore the normal rate

of the T7 DNA synthesis.

To examine whether or not the first round of replication of the

UP-2 mutant occurs on 594ssb-1, the bacteria which had grown in a

medium containing BrdU, were infected with ^{32}P-labelled UP-2 or ^{32}P-

labelled wild-type phage, and incubated at 37°C for 15 min. DNA

molecules were extracted and centrifuged in a CsCl equilibrium density

gradient (Fig. III-5). In the case of T7 wild-type phage, about 50%

of total radioactivity was recovered in the half-heavy portion (Fig. III-

5A). However, less than 1% of total radioactivity was recovered in

the half-heavy portion in the case of T7 UP-2 phage (Fig. III-5B).

So even the first round of replication does not occur with the T7 UP-2

mutant. This result shows that T7 DNA-binding protein participates
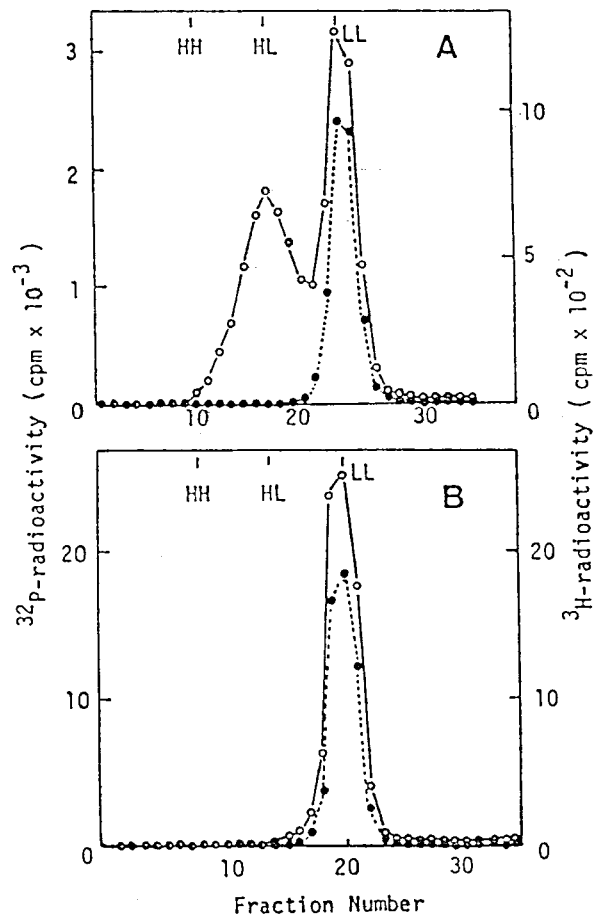
Figure III-5.   Density labelling experiment of replication of T7 wild-type and T7 UP-2 mutant in 594ssb-1 host bacteria.

Bacteria, 594ssb-1, were grown to 1 x 10^8 cells/ml in Cas-λ broth containing 6 µg thymine/ml, harvested by centrifugation, resuspended in Cas-λ broth containing 10 µg BrdU/ml and 1 µg thymine/ml and incubated. When the cells were grown to 2 x 10^8/ml, ^{32}P-labelled T7 wild-type (A) or UP-2 (B) phages were added at a multiplicity of 10 and incubated at 37°C for 15 min.   Centrifugation was carried out in a Spinco 40 rotor at 36,000 revs/min for 40 hr at 15°C. ^{3}H-labelled T7 DNA was added to determine the position of the light DNA.   ——o—— ^{32}P-radioactivity, ------●------ ^{3}H-radioactivity

in the DNA replication of T7 phage, at least, in the first round.

Effect of the UP-2 mutation on recombination frequency

To understand the effects of the T7 DNA-binding protein mutation on genetic recombinaiton, an amber mutation, am233 (gene 6) or am10 (gene 19) was inserted into this mutant UP-2 phage. Using the two doubled mutants UP-2am233 and UP-2am10 obtained, two points crosses were performed. As shown in Table III-5, in ssb$^+$ or ssb-1 bacteria, recombination frequency of T7 wild-type phage between am233 and am10 mutation is around 30%. However, when using the UP-2 mutant phage, the same cross was carried out in 594ssb-1 bacteria, the recombinaiton frequency was reduced to about 4%. In this cross, a few phage bursts were observed (about 3 per cell), although the plaque of this mutant was not seen on this host. This reduction in recombination frequency was also observed even if the UP-2 mutation was suppressed in Q1 bacteria. This distinction between DNA synthesis and recombination under the suppressed condition will be discussed later (see Discussion). Moreover, when recombination was examined with host bacteria harboring the plasmid pDR1996 containing the ssb gene of E. coli, which produce more than 10-fold the normal level of E. coli DNA-binding protein (W. D. Rupp, personal communication), no significant increase in the recombination frequency was observed. Thus DNA-binding protein of E. coli cannot substitute for this UP-2 mutant protein in the T7 genetic recombination process.

Effect of the UP-2 mutation on the formation of the intermediate DNA molecules of genetic recombination

Tsujimoto and Ogawa (1977, 1978) showed that suppressor free bacteria were infected with both BrdU-labelled T7 phage and ^{32}P-labelled T7 phage

Table III-5

Effect of the UP-2 mutation on recombination frequency

Cross	host strain[a]	burst size	recombination frequency
am233 x am10	Q1	119	25.3(%)
	594ssb-1	15	36.5
	Q1(pDR1996)	60	26.1
UP-2am233	Q1	35	3.1
x	594ssb-1	3	4.1
UP-2am10	Q1(pDR1996)	40	3.7
r-2am233 x r-2am10	Q1	225	24.4
	594ssb-1	72	27.5

The procedures were described in MATERIALS AND METHODS. The number of recombinants was obtained by doubling the number of plaques on 594 after correcting for the plating effciency relative to Q1. The plating efficiencies of T7 wild-type, UP-2 and r-2 on 594 were 0.75, 0.46 and 0.96, respectively. Recombination frequency is expressed as a percentage of the recombinants among the total plaques on Q1. T7am233 is an amber mutant in gene 6 and T7am10 phage is that in gene 19.

a. A strain used in a cross.

which carried mutiple amber mutations in genes for DNA replication, genes

$\underline{2}$, $\underline{3}$, $\underline{4}$, and $\underline{5}$ about 1.2% of the input ^{32}P counts were banded at the

position of an intermediate density of two parental DNA.   Most of

these molecules had a letter H-like configuration and were demonstrated

to consist of two intact parental T7 DNA molecules that were fused in

the interval between two branch points.   Furthermore, these molecules

were infective and could yield recombinants at high frequency.

Therefore, these intermediate density molecules formed in the absence of

DNA replication were consisted to be the intermediates of genetic

recombination.   To see the effect of the UP-2 mutation on the

formation of these intermediate density molecules, I first tried to

construct a phage carrying multiple amber mutations in genes for DNA

replication in addition to the UP-2 mutation.   This was unsuccessful

for unknown reasons.   Therefore, I used a host defective in

thioredoxin ($\underline{trx}$) which is a subunit of T7 DNA polymerase to prevent

the synthesis of T7 DNA instead of using T7 mutants.

Bacteria, 594$\underline{trxAssb-1}$, were infected with ^{32}P- and BrdU-labelled

T7 phages carrying the UP-2 mutation and incubated for 15 min at 37°C.

DNA molecules were extracted and centrifuged in a CsCl equilibrium

density gradient (Fig. III-6d).   Fractions 13 to 17 in Figure III-6d,

which were heavier in density than the light DNA, were pooled and recentrifuged

in a CsCl equilibrium density gradient.   0.8% of the total input

radioactivity was unexpectedly recovered in the half-heavy portion

(Fig. III-6e).   However, when peak fractions in the half-heavy portion

were dialyzed and treated with RNase, almost all radioactivity was

shifted to the light position in a CsCl equilibrium density (Fig. III-

6f).   Therefore, the intermediate density DNA molecule which was

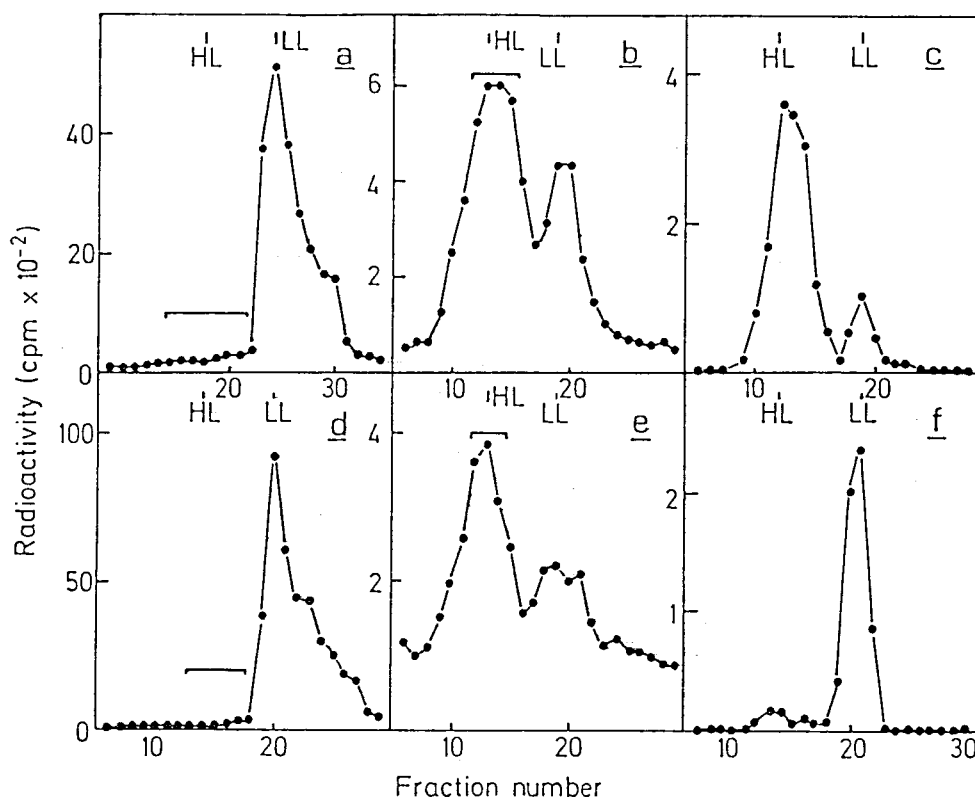resistant to RNase was not found in the T7 UP-2 infected cell extract.

Figure III-6.   Formation of intermediate density molecules in 594trxAssb-1
bacteria after infection with ^{32}P-labelled and BrdU-labelled UP-2 mutant phage.

The 594trxAssb-1 bacteria were infected with ^{32}P- and BrdU-labelled
T7 UP-2 mutant phages each at a multiplicity of infection of 20, and
incubated at 37°C for 15 min.   The infected cells were lysed, treated with
sodium N-lauroyl sarcosinate-Pronase and then the lysates were centrifuged
in a CsCl density gradient.   As a control, the same experiment was performed
using T7 wild-type phage simultaneously.   (a) Centrifugation profile of
DNA molecules extracted after T7 wild-type phage infection.   (b) Recentri-
fugation profile of the pooled fractions, 15 to 21 in (a).   (c) CsCl
density gradient centrifugation of peak fractions, 12 to 15 in (b).
Before the centrifugation, the peak fractions were pooled, dialyzed against
SSC containing 2 mM EDTA for 2 hr at 0°C, diluted 2-fold with a water,
and treated with 100 µg RNase A/ml for 2 hr at 20°C.   (d) Centrifugation
profile of DNA molecules extracted from the cells infected with T7 UP-2
phage.   (e) Recentrifugation profile of the pooled fractions, 13 to 17 in
(d).   (f) A profile of CsCl density gradient centrifugation of peak fractions,
12 to 14 in (e).   Before the centrifugation, the peak fractions were
pooled and treated similarly as described in (c).

Centrifugation was carried out in a Spinco 40 rotor at 36,000 revs/min
for 40 hr at 15°C.   The position of the peak of the light DNA was determined
by ^{3}H-labelled T7 phage DNA added before centrifugation, and the position
of half-heavy and full heavy DNA were deduced from the density of BrdU-
labelled T7 DNA.   HH: fully heavy, HL: half-heavy, LL: fully light.

When the same experiment was carried out by using wild-type T7 phage, 2.4% of total radioactivity was recovered in the half-heavy portion (Fig. III-6a, b).   In contrast to the molecules extracted after T7 UP-2 infection, 80% of the DNA molecules in the half-heavy fractions remained at the half-heavy density after treatment with RNase (Fig. III-6c).   The DNA at the half-heavy position that was shifted to the light position by RNase treatment is probably a DNA-RNA hybrid molecule, because similar amount of the ^{32}P-labelled DNA was recovered at the half-heavy position from the mutant host without BrdU-labelled phage infection and the half-heavy molecules were shifted to the light position by RNase treatment (data not shown).   Observation of similar DNA-RNA hybrid molecules was reported also with the φX174 (Baas et al., 1978) and λ phage-infected cells (Masukata et al., 1979).

Effect of the UP-2 mutation on UV sensitivity of T7 phage

    DNA-binding protein seems to also participate in the repair of DNA damage, since bacterial mutant, ssb-1 is extremely sensitive to UV irradiation (Glassberg et al., 1979).   The UV sensitivity of T7 UP-2 mutant was examined on ssb-1 mutant bacteria and wild-type bacteria. The survival curves of the mutant phage and wild-type phage are shown in Fig. III-7.   The mutant UP-2 was found to be 1.3 - 1.9 times more sensitive than the wild-type on 594 (Fig. III-7B) and JC1557 (Fig.III-7C).   The ratio of the sensitivity of the T7 UP-2 mutant to that of T7 wild-type phage is almost the same as the ratio of T7 wild-type phage infectivity of uvrA mutant bacteria compared to wild-type bacteria (Fig. III-7C).   This difference in UV sensitivity between wild-type phage and the UP-2 mutant was also observed even on C600ssb-1 and Q1 bacteria, regardless of the presence or absence of the ssb-1 mutation.
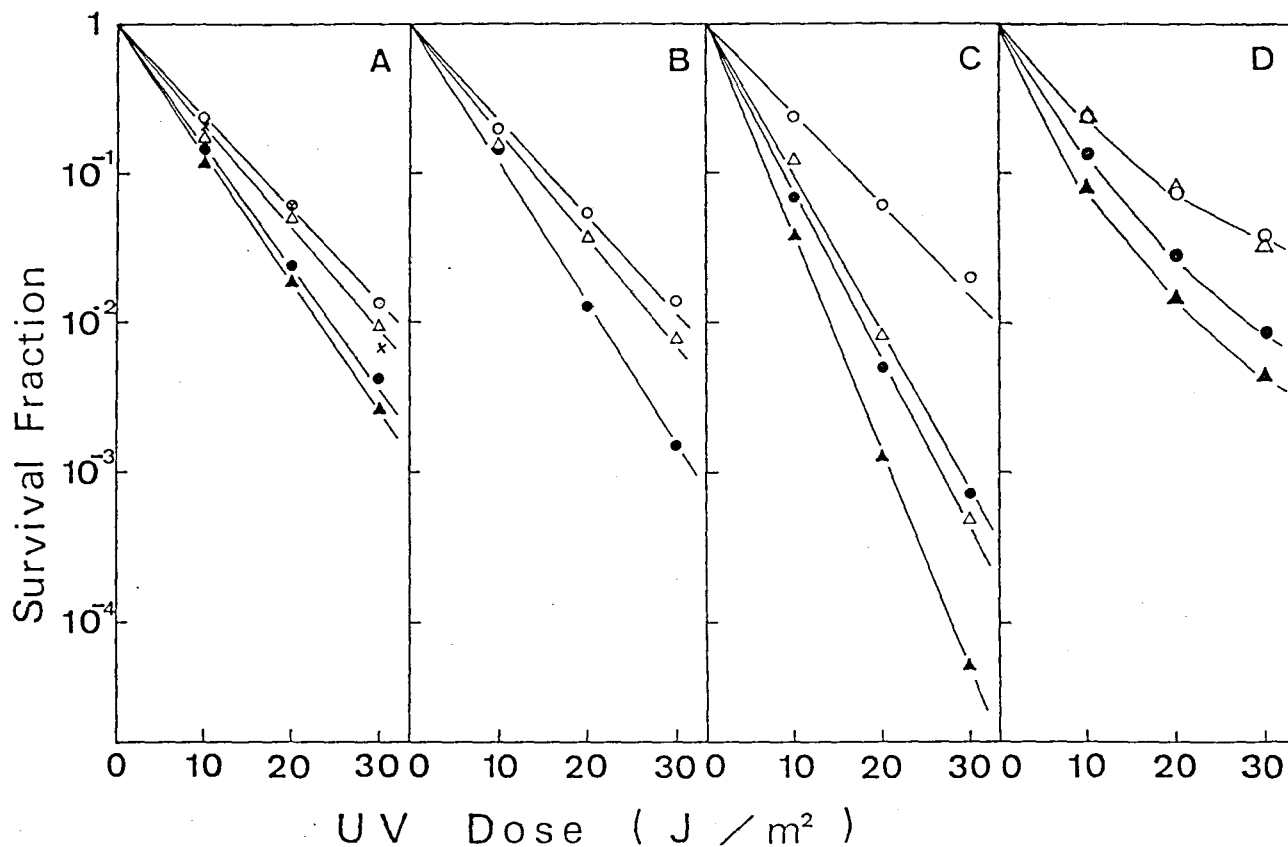
Figure III-7.   UV sensitivity of T7 UP-2 phage

T7 wild-type, UP-2 mutant and r-2 revertant phage were irradiated with UV light and plated with various indicator strains.   The open symbols are the survival fractions of T7 wild-type, the closed symbols are those of T7 UP-2, and the crosses are those of T7 r-2.   Indicator strains are Q1 (o●x) and C600ssb-1(Δ▲) in (A), 594 (o●) and 594ssb-1 (Δ) in (B), JC1557 (o●) and JC1557uvrA (Δ▲) in (C), 594(pDR1996) (o●) and 594 (Δ▲) in (D).

(Fig. III-7A). Besides, the UP-2 mutant was still more sensitive

than the wild-type phage by a factor of 1.3 on bacteria 594(pDR1996)

which have increased amounts of E. coli DNA-binding protein, although

it was slightly more resistant than that on its parental strain 594

(Fig. III-7D). The survivals of the wild-type phage were unaffected

by this 594(pDR1996) strain (Fig. III-7D). A revertant of the UP-2

mutant, r-2, completely restored the repair ability (Fig. 6A). These

results suggest that T7 DNA-binding protein is itself involved in T7

DNA repair as well as in DNA replication and in T7 recombination, and

that the bacterial DNA-binding protein does not substitute effectively

for T7 DNA-binding protein in the process of repair of UV-damaged T7 DNA.

DISCUSSION

A T7 mutant phage, UP-2, which was isolated from mutants incapable

of growing on 594ssb-1 cells, was defective in T7 DNA-binding protein

and synthesized a smaller polypeptide than T7 wild-type protein.

The gene that codes for T7 DNA-binding proetin was mapped between gene 2

and gene 3, and referred to as gene 2.5.   This map position is not

consistent with the location tentatively determined by Hausman, who

placed the gene between gene 3.5 and gene 4.   Recently, Dunn and

Studier (1981) determined the nucleotide sequence between left end and

the right portion of gene 4 of T7 phage genome and they found the gene

for T7 DNA-binding protein between gene 2 and gene 3 .

In an in vitro system of T7 DNA synthesis, E. coli DNA-binding protein

is suggested to be able to substitute for T7 DNA-binding protein (Reuben

& Gefter, 1974; Scherzinger & Klotz, 1975).   In contrast with these

in vitro results, the ability of the UP-2 mutant phage for DNA synthesis

is very low even in 594 bacteria.   When the mutation is suppressed in

a permissive host, the replicating ability of the mutant phage recovered

to 60 - 70% level of that of the wild-type phage.   Thus, E. coli DNA-

binding protein is unable to function well as a substitute for T7 DNA-

binding protein in in vivo T7 DNA replication.   The ability of E. coli

DNA-binding protein to replace T7 DNA-binding protein was not observed

on recombination and UV-repair of T7 DNA.   The efficiencies of recombination

and UV-repair in UP-2 infected cells do not increase even in the bacteria

carrying pDR1996 plasmid, which syntheisze about 10 times the amount of

E. coli DNA-binding protein.   These results suggest that the inefficiency

of E. coli DNA-binding protein to substitute for T7 UP-2 mutant protein

is not due to less amount of E. coli DNA-binding protein in the cell

but due to a functional difference between two proteins.   Although

the UP-2 mutant phage can grow in C600 cells, the efficiencies of

genetic recombination and DNA-repair are very low.   The suppression

of UP-2 phage in C600 strain is mysterious since UP-2 is an opal mutant

(see Chapter IV) and C600 strain have an amber suppressor.   This

problem will be explained in the next part (Chapter IIIB).

Recombination intermediate molecules of the UP-2 DNA were not

found at all in 594ssb-1 bacteria.   Tomizawa et al. (1966) has shown

that recombination intermediate was not formed in the cells infected

with T4 mutant phage defective in the DNA-binding protein coded by

gene 32.   These results suggest that the participation of the DNA-

binding protein in the formation of joint molecules between two parental

DNAs is general in recombination process.

T7 mutants isolated, which could not grow on 594ssb-1 cells, were

divided into 7 different complementation groups.   One of them is the

UP-2 mutant phage.   They carry mutations in genes involving transcription

(gene 1), DNA metabolism (genes 2.5, 3, 3.5) and morphogenesis (genes

12, 14, 16).   This isolation method of the mutants should be useful

for obtaining an insight into the process in which E. coli DNA-binding

protein is involved.

A new T7 mutant defective in DNA replication was reported by North

and Molineux (1980).   The mutation has been mapped between gene 2 and

gene 3 and separated from a mutation in T7 DNA-binding protein,

which results in production of a protein 10% smaller than the wild-type

protein.   The discrepancy between their results and me remains unknown.

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III-B

FURTHER CHARACTERIZATION OF T7UP-2 PHAGE

ABSTRACT

As a UP-2 strain is found to be an opal mutant, further characterization
of UP-2 strain using CAJ64 bacteria harboring a strong opal suppressor
carried out.   The abilities of recombination and repair of a UP-2 strain
are suppressed completely by this suppressor in contrast with the case on
Q1 bacteria which has a weak opal suppressor.   However, for DNA synthesis,
the delay of the synthesis was still observed in this strain as observed
on Q1 bacteria.   The UP-2 mutant was found to have another mutation
outside the gene 2.5, which seems to suppress the defect of the gene 2.5.
The UP-2 mutant free from this suppressor mutation can grow on Q1 bacteria
but not on 594 bacteria.   Thus, T7 DNA-binding protein cannot be replaced
by E. coli DNA-binding protein in replication, recombination and repair.

T7UP-2 strain, which was thought to be an amber mutant in the gene 2.5 from isolation procedures, was found to be an opal mutant in the same gene by analysis of the nucleotide sequence (Chapter IV). In this part, the reasons why a UP-2 strain could be isolated will be explained and properties of a UP-2 strain are also examined using bacteria CAJ64 (Sambrook et al., 1967) having a strong opal suppressor.

One possibility of the isolation of a UP-2 opal mutant, is that bacteria, Q1 and C600 used for the isolation have an opal suppressor in addition to an amber suppressor. To examine this possibility, the opal mutants of bacteriophage T4 (eL1 and eL5) which were obtained from Dr. H. Inokuchi were titrated with Q1, C600 and CAJ 64. The plating efficiencies of eL1 and eL5 on Q1 and C600 bacteria were 10^{-1}-10^{-2} of those on CAJ64 bacteria, while those on 594 bacteria were about 10^{-5}. This result indicates that bacteria strains Q1 and C600, have a weak opal suppressor. A weak suppressor of C600 bacteria should make small amount of wild-type T7 DNA-binding protein (Fig. III-2). This is one reason why a UP-2 mutant could be isolated.

Previously I tried to construct a double mutant which carries a UP-2 and the mutation in the genes of DNA synthesis but failed it from unknown reasons (Chapter IIIA). However, if in a UP-2 mutant there is an another mutation outside the gene 2.5 necessary for the growth of a UP-2 strain in the condition of weak suppression or no suppression, the failure of construction of double mutant described above may be explained. To test this possibility, using CAJ64 as host bacteria, a UP-2 mutant was crossed with wild-type T7 phage to eliminate a hypothesized outside mutation. Then an opal suppressor-sensitive phage strain (op-1) could be isolated and its mutation was mapped between gene 2 and gene 3. Therefore, op-1

mutation should be occurred in the gene 2.5. And the T7 strain having an op-1

mutation and mutation in the genes of DNA synthesis could be isolated

using bacteria having both an opal and an amber suppressor.   An op-1 strain

made minute plaques on Q1 bacteria at the same plating efficiency as

that on CAJ64 bacteria but the plating efficiency of an op-1 strain on 594

was decreased by 10^{-4}.   This evidence suggests that a UP-2 strain has an

another suppressor mutation which makes a UP-2 strain able to grow on

suppressor free or weak suppressor host bacteria.   Mosig et al. (1978)

reported that bacterial proteins which is not used by a T4 wild-type

phage are used by a T4 mutant phage in DNA-binding protein.   An outside

mutation cannot be mapped easily as it is not lethal.   These results

suggest that a UP-2 mutant could be isolated by two reasons; bacteria Q1

and C600 used for the isolation have a weak opal suppressor and a UP-2

strain has a suppressor mutation in addition to an opal mutation in the gene

2.5.

As a UP-2 phage is found to be an opal mutant, the effect of a strong

opal suppressor on a UP-2 mutant was examined using CAJ64 bacteria.

Plating efficienciy of a UP-2 mutant on CAJ64 is the same as that on Q1.

DNA synthesis was examined by pulse-labelling experiments.   As shown

in Fig. III-8, the onset of DNA synthesis of a UP-2 strain in CAJ64 bacteria

was delayed and the maximum rate of incorporation was slightly lower than

that of wild-type T7 phage (Fig. III-4).   This delay in DNA synthesis

was also observed in the op-1 mutant which is free from a suppressor

mutation outside the gene 2.5 .   Therefore, an outside mutation seems

not to be concerned with the delay of DNA synthesis.   These results

suggest that small amount of T7 DNA-binding protein seems to be enough
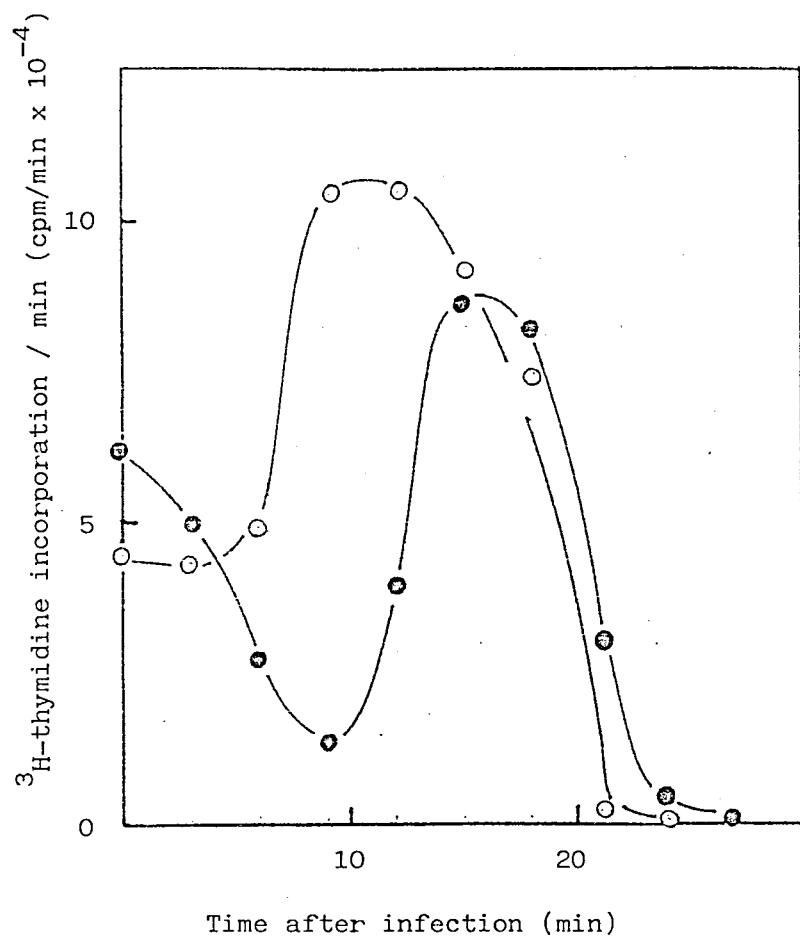
Figure III-8.   Time course of the rate of the DNA synthesis after the

infection of T7 phage.

Bacteria, CAJ64, were infected with T7 wild-type(O) or UP-2 (⊙) at

37°C.   Procedures are the same as those described in the legend of

Figure III-4.

to carry out for T7 DNA synthesis although DNA synthesis delayed significantly. But it cannot be denied that the delay of DNA synthesis is caused by an other mutation closely linked to the gene 2.5. Recombination frequency and UV sensitivity of a UP-2 strain were also examined using CAJ64 bacteria. As shown in Table III-6 and Fig.7, deficiencies of a UP-2 strain in recombination and repair were suppressed almost completely.

From the results described in this part, it is concluded that Q1 and C600 have a weak opal suppressor, T7UP-2 seems to have an additional suppressor mutation for an opal mutation in the gene 2.5 at least and T7 DNA-binding protein cannot be replaced by E. coli DNA-binding protein in replication, recombination and repair in contrast with in vitro results (Reuben and Gefter, 1974; Scherzinger and Klotz, 1975).

Table III-6

Recombination frequency of a UP-2 mutant in CAJ64 bacteria

Crosses	Burst size	Recombination frequency (%)
UP-2am233 X UP-2am10	38	29.2
am233 X am10	101	38.3

The procedures are described in the legend of Table III-5.
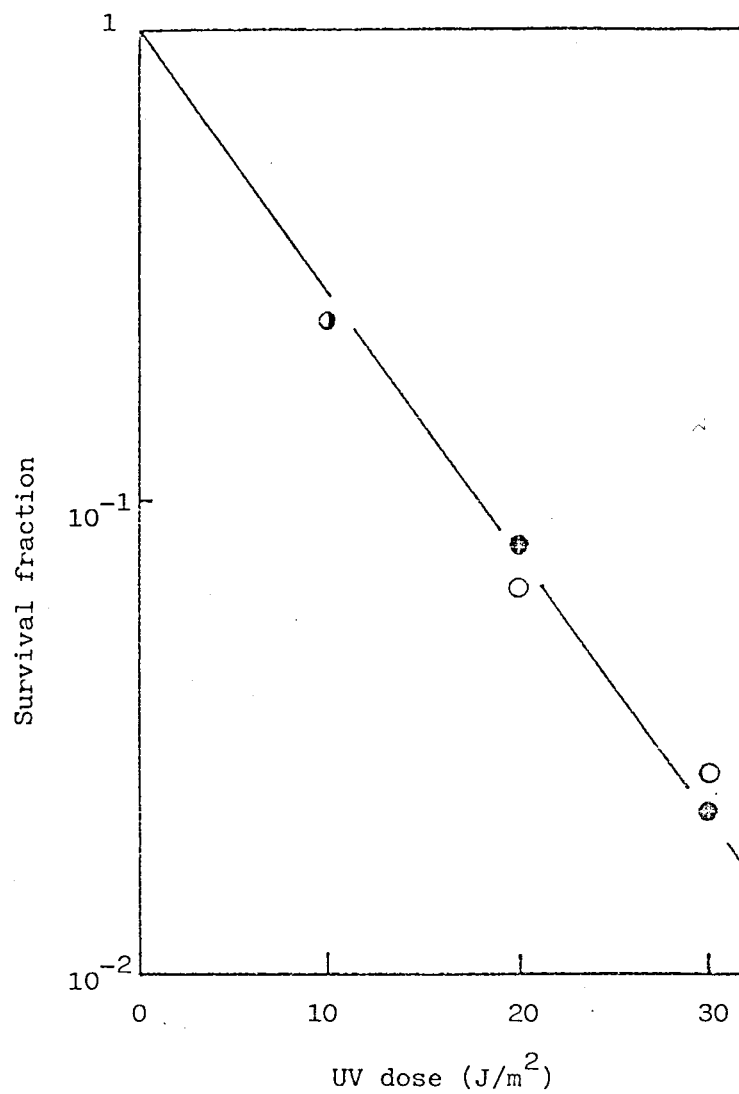Bacteria, CAJ64, were used for recombination.

Figure III-9.   UV sensitivity of T7 UP-2 phage on CAJ64 bacteria.

T7 wild-type (O) and UP-2 mutant (⊕) were irradiated with UV light

and plated with CAJ64 bacteria.

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IV

ISOLATION AND CHARACTERIZATION OF · T7 MUTANT DNA-

BINDING PROTEIN SYNTHESIZED BY T7UP-2 PHAGE

ABSTRACT

Analysis of nucleotide sequence of the gene 2.5 of T7UP-2 mutant

revealed that nucleotide G on 1 strand at nucleotide 9,805 of the gene

changed to A, and that the codon changed from tryptophan to nonsense,

opal, by that mutation.   Hence it was predicted that the mutant DNA-

binding protein probably lacks 17 amino acids at the carboxyl terminal

region.   The mutant DNA-binding protein was purified and characterized

in comparison with wild-type T7 DNA-binding protein.   And the following

facts were revealed.   1) Amino acid composition and the sequence of 5

amino acids at amino terminus of the purified mutant protein were

the same as the predictions from the nucleotide sequence.   2) Mutant

protein binds to single-stranded DNA more efficiently than does wild-type

protein even at a low concentration and can bind to double-stranded DNA

at far less concentration than the wild-type protein.   3) Two activities

of T7 mutant DNA-binding protein, renaturation of homologous single-

stranded DNA and stimulation of exonuclease activity, are inefficient at

the optimal concentration for wild-type protein.   4) Mutant protein can

unwind native double-stranded DNA although wild-type protein cannot do.

5) Carboxyl terminal region of T7 DNA-binding protein seems to be required

for interaction with T7 replication enzyme since mutant protein cannot

stimulate the replication of double-stranded DNA in contrast with wild-

type protein.   6) These results suggest that carboxyl terminal region

of T7 DNA-binding protein controls its activities.

INTRODUCTION

Single-stranded DNA-binding proteins have been isolated from many organisms (Champoux, 1978). They have been thought to play important roles in the metabolism of the genetic materials. Bacteriophage T7 also encodes its own single-stranded DNA-binding protein (Reuben and Gefter, 1973). Purified T7 DNA-binding protein stimulates in vitro DNA synthesis (Reuben and Gefter, 1973; Scherzinger et al., 1973; Scherzinger and Klotz, 1975; Richardson et al., 1978) and the pairing process of the parental DNAs in in vitro genetic recombination (Sadowski et al., 1980; Araki and Ogawa, 1981a). The participation of T7 DNA-binding protein in replication and the pairing process of recombination in vivo has been proven by the isolation of a T7 mutant phage, UP-2, defective in T7 DNA-binding protein (Araki and Ogawa, 1981b). T7UP-2 phage which synthesized a 10% smaller polypeptide than T7 wild-type DNA-binding protein, has the reduced abilities of DNA replication, recombination and repair. T7UP-2 phage cannot form the pairing DNA molecules in genetic recombination and cannot carry out even first round replication of T7 DNA. From the analysis of a deletion mutant in T7 DNA-binding protein, which has shown normal DNA synthesis, it has been suggested that T7 DNA-binding protein might participate in transcription (Yeats et al., 1981).

Besides the activities described above, T7 DNA-binding protein has renaturation activity of homologous single-stranded DNA and stimulating activity for exonucleases (Sadowski et al., 1980). But it has been ambiguous what activities mentioned above are involved in recombination or replication. Comparative study between the mutant protein coded by T7 UP-2 phage and wild-type T7 DNA-biding protein would give us more

informations about such question.   The mutant DNA-binding protein coded

by T7UP-2 phage expected to lack the carboxyl terminal region of T7 DNA-

binding protein, since the mutation is a nonsence mutations near the

carboxyl terminus.   Proteolytic removal of the carboxyl terminal portion

of DNA-binding protein coded by bacteriophage T4 and Escherichia coli

has suggested that the carboxyl terminal portion might play a role of

regulation of its activity (Moise and Hosoda, 1976; Williams et al.,

1981).   Therefore, it is interested whether or not the carboxyl

terminal portion of T7 DNA-binding protein plays a similar role of regulation

of its activity.   Moreover, recently the nucleotide sequence of gene

2.5 coding for T7 DNA-binding protein was determined by Dunn and Studier

(1981) and hence the amino acid sequence.   These reasons have prompted

me to isolate and characterize the mutant protein in comparison with

wild-type T7 DNA-biding protein.

As expected, the purified mutant T7 DNA-binding protein has shown

altered activities in replication, renaturation of DNA, stimulation of

T7 exonuclease activity, and DNA binding.   From these results, the

functions involved in replication and genetic recombination and the

role of carboxyl terminal region will be discussed.

MATERIALS AND METHODS

Preparation of labelled DNA

^{3}H-labelled T7 DNA was prepared as described by Tsujimoto and Ogawa

(1977).　Preparation of ^{3}H-labelled φx174 DNA and ^{3}H-labelled φx174

RFI DNA was carried out according to the procedure of Pagano and Huchinson

III (1971).

Purification of mutant T7 DNA-binding protein

All procedures were carried out at 0-4°C and centrifugations were at

15,000 g for 30 min unless otherwise indicated.　Protein concentration

was determined by the method of Lowry et al. (1951).　The purification

of the mutant protein was carried out by examining its amount with sodium-

dodecylsulfate(SDS) polyacrlamide gel electrophoresis.　The identification

of the purified protein was shown by determining its amino acid composition

and amino acid sequence at amino terminal region since both were predicted

from the nucleotide sequence (Dunn and Studier, 1981).

T7UP-2 phage (Araki and Ogawa, 1981b;Chapter III) was added at a

multiplicity of 5, to 12-liter culture of 594endA bacteria (Ogawa et al.,

1978) grown to 10^9 cells/ml at 37°C in L-broth (Ikeda and Tomizawa, 1965).

After incubation at 37°C for 18 min, the infected cells were harvested

by centrifugation (10,000 g for 10 min) at 0°C.　The cells were suspended

in 120 ml of 50 mM Tris-HCl (pH. 7.4) containing 10% sucrose and frozen in

a dry ice/acetone bath.　Frozen cells (175 ml) were thawed at 0°C and 3.5

ml each of 10 mg/ml of lysozyme and 5 M NaCl were added.　After 45 min at

0°C, the solution was stirred gently in a hot water bath to bring the

temperature to 20°C and incubated at 20°C for further 10 min.　It was

then transferred to an ice-bath and stirred until the temperature reached 10°C. The lysate was centrifuged for 25 min at 25,000 rpm in a Beckman type 30 rotor. The supernatant fluid was adjusted to A_{260}=200 by the addition of 50 mM Tris-HCl (pH. 7.4) containing 10% sucrose and 0.1 M NaCl (167 ml), and then 17 ml of 40% streptomycin sulfate was added. The solution was stirred at 0°C for 30 min and the precipitate was removed by centrifugation. To 180 ml of the supernatant, was added 63 g of ammonium sulfate. After stirring at 0°C for 45 min, the precipitate was collected by centrifugation and dissolved in 20 ml of buffer AP (20 mM potassium-phosphate buffer (pH. 7.4), 0.1 mM **EDTA**, 1 mM 2-mercaptoethanol, 10% glycerol) containing 0.2 M KCl. The suspension was dialyzed against the same buffer overnight and applied to a column of Brown DEAE-cellulose (3.5 cm^2 x 24 cm) equilibrated with buffer AP containing 0.2 M KCl. Breakthrough fractions (140 ml) were mixed with the same volume of buffer AP and loaded on a column of Whatman P11 phosphocellulose (3.5 cm^2 x 20 cm) equilibrated with buffer AP containing 0.1 M KCl. The column was washed with 250 ml of buffer AP containing 0.1 M KCl and the proteins bound to the column were eluted with buffer AP containing 0.5 M KCl. After the fractions of 0.5 M KCl wash were pooled (114 ml), the proteins were precipitated with 51.3 g of ammonium sulfate and collected by centrifugation. The pellet was dissolved in 10 ml of standard buffer (20 mM Tris-HCl (pH. 7.4), 1 mM 2-mercaptoethanol, 10% glycerol) containing 0.2 M NaCl and the suspension was dialyzed against the same buffer. The dialyzed sample was applied to a column of single-stranded DNA-cellulose (2 cm^2 x 15 cm) which was prepared as described previously (Araki and Ogawa, 1981a; Chapter II) and equilibrated with standard buffer containing 0.2 M NaCl. The column was washed with 150 ml of

standard buffer containing 0.2 M NaCl and the bound proteins were eluted

with standard buffer containing 1.0 M NaCl.   The eluted protein fraction

were pooled and dialyzed against standard buffer containing 0.2 M NaCl.

The sample was mixed with the same volume of standard buffer and applied

to a column of DEAE-sephadex-A25 (1.1 cm^2 x 15 cm) equilibrated with

standard buffer containing 0.1 M NaCl.   The column was washed with 20 ml

of standard buffer containing 0.1 M NaCl and the proteins were eluted with

a 160 ml linear gradient from 0.1 to 0.5 M NaCl in standard buffer.

The mutant DNA-binding protein was eluted between 0.16 and 0.2 M NaCl.   The

fractions between 0.16 and 0.20 M NaCl were pooled (16 ml) and dialyzed

overnight against 50 mM Tris-HCl (pH. 7.4) saturated with ammonium sulfate.

The precipitate was collected by centrifugation and suspended with 0.8

ml of standard buffer containing 1.0 M NaCl.   The suspension was passed

through a sephadex G-100 column (2 cm^2 x 40 cm) equilibrated with standard

buffer containing 1.0 M NaCl.   The peak of the mutant DNA-binding protein

appeared at 45% of the column volume.   Peak fractions (19 ml) were

pooled and dialyzed against buffer AP containing 0.1 M NaCl.   This

fraction was used for determining amino acid composition and amino terminal

amino acid sequences.   About 3 mg of mutant DNA-binding protein which

was more than 90% pure was recovered.   To concentrate the mutant DNA-

binding protein, a half of the dialyzed sample was adsorbed to phospho-

cellulose column (0.5 cm^2 x 2 cm) equilibrated with buffer AP containing 0.1

NaCl and eluted with 3 ml of buffer Ap containing 0.5 M NaCl.   The concentrated

sample was dialyzed against standard buffer containing 0.15 M NaCl and

used for the other analyses.

Wild-type T7 DNA-binding protein , T7 exonuclease, T7 DNA polymerase, and T7 primase

Purification of T7 exonuclease and wild-type T7 DNA-binding protein was described by Shimizu et al. (1982).   T7 DNA polymerase and T7 primase are gifts of Dr. K. Hori.

Assay for binding to DNA

Reaction mixture (0.1 ml) contained 20 mM Tris-HCl (pH. 7.4), 10 mM $MgSO_4$, 1 µg/ml ^{3}H-labelled ϕx174 phage DNA or ϕx174 RFI DNA, and T7 DNA-binding protein at indicated concentration.   After incubation at $30^{\circ}C$ for 10 min, the sample was passed through nitrocellulose filter (millipore HA).   The filter was washed with 0.1 ml of 20 mM Tris-HCl (pH. 7.4) containing 10 mM $MgSO_4$.   The radioactivity retained on the filter was measured in a liquid scintillation counter.

Assay for renaturation and denaturation of DNA

Reaction mixture contained 20 mM Tris-HCl (pH. 7.4), 0.1 M NaCl, 2 µg/ml ^{3}H-labelled T7 DNA, T7 DNA-binding protein at indicated concentration and 10 mM $MgSO_4$ if indicated.   Heat-denatured ^{3}H-labelled T7 DNA was used for renaturation assay and native ^{3}H-labelled T7 DNA for denaturation assay.   Reaction mixtures were incubated at $30^{\circ}C$ for the indicated time. 25 µl portion each of reaction mixture (containing about 2,000 cpm radioactivity) was then mixed with 2.5 µl of 10% SDS and incubated at $30^{\circ}C$ for 1 min further.   A half milliliter of 0.1 M sodium-acetate buffer (pH. 4.3) containing 0.3 M NaCl, 0.1 mM $ZnSO_4$ and 0.02% SDS was added with 1,000 units of S1-nuclease (Sankyo) to SDS-treated sample, and the mixture was incubated at $30^{\circ}C$ for 20 min.   A half milliliter of chilled 10% trichloroacetic acid was poured into a S1-treated sample.

The sample was filtered through a nitrocellulose filter, washed with 5% trichloroacetic acid, and the radioactivity retained on the filter was measured in a liquid scintillation counter.   S1-nuclease-sensitive fraction was calculated by subtracting the radioactivity retained from the input radioactivity.

Stimulation of T7 exonuclease activity by T7 DNA-binding protein

Reaction mixture (50 μl) contained 50 mM Tris-HCl (pH. 8.0), 20 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10 μg/ml ^{3}H-labelled T7 DNA (15,000 cpm), 0.1 unit of T7 exonuclease and T7 DNA-binding protein at indicated concentration.   After incubation at 30°C for 30 min, 25 μl of 2 mg/ml yeast DNA (Kojin) and 75 μl of 2 N HCl were added to the reaction mixture. The mixture was centrifuged and the supernatant was counted in a liquid scintillation counter.

Stimulation of in vitro DNA synthesis by T7 DNA-binding protein

Stimulation of DNA polymerase   of the procedure of Grippo and Richardson (1971).   Reactions (0.1 ml) contained 88 mM potassium-phosphate buffer (pH. 7.4), 6.7 mM $MgCl_2$, 5 mM 2-mercapto-ethanol, 5 μg/ml heat-denatured T7 DNA, 0.15 mM concentrations of dATP, dGTP, dCTP and ^{3}H-dTTP (50 cpm/pmol), 0.2 unit of T7 DNA polymerase, and T7 DNA-binding protein at indicated concentration.   Incubation was at 15°C for 30 min and terminated by the addition of 20 μl of 1 mg/ml BSA and 0.4 ml of 1 N HCl, 0.1 M pyrophosphate.   The sample was filtered through a glass filter and washed with 10 ml of 1.N HCl, 0.1 M pyrophosphphate The filter was dried and counted in a liquid scintillation counter.

Stimulation of replication of duplex T7 DNA was assayed by a modification of the procedure of Kolodner et al. (1978).   Reactions (0.1 ml) contained

40 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 0.3 mM

each of ATP, UTP, CTP and GTP, 0.3 mM each of dATP, dGTP, dCTP and ^{3}H-

dTTP (50 cpm/pmol), 5 nmols of T7 DNA, 0.1 unit of T7 DNA polymerase,

1.7 unit of T7 primase, and T7 DNA-binding protein at indicated

concentration.   After incubation at 30°C for 20 min, the acid-insoluble

radioactivity was determined as described above.

Gel electrophoresis

SDS-polyacrylamide gel electrohporesis was carried out according to

Studier (1973).   The isoelectric focusing was carried out according to

O'Farrel (1975).

Electron microscopy

φx174 DNA (5 µg/ml) was incubated with 100 µg/ml T7 DNA-binding protein

at 30°C in 10 mM potassium-phosphate buffer (pH 7.0) for 10 min.

The sample was fixed at 30°C with 0.1 M glutaraldehyde (Polysciences, EM

grade) for 15 min and spread by the formamide technique described by

Davis et al. (1971).

Determination of nucleotide sequence

After cleaving T7UP-2 DNA by KpnI and AvaI restriction endonucleases,

the KpnI-AvaI fragment (nucleotide 9,193-10,512) was isolated by polyacryl-

amide gel electrophoresis and was further cleaved by AluI or HinfI

restriction endonuclease.   The AluI fragment (nucleotide 9,802 - 10,199)

or the HinfI fragment (nucleotide 9,675 - 9,821) was isolated by polyacryl-

amide gel electrophoresis and the 5' ends of fragments were labelled with

^{32}P by using T4 polynucleotide kinase.   After cleaved by HhaI (for the

AluI fragment) or TaqI (for the HinfI fragment) endonuclease, the HinfI-

TaqI fragment (nucleotide 9,691-9,821) and the AluI-HhaI fragment (

nucleotide 9,802 - 9,926) 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).

Determination of amino acid composition, amino terminal amino acid sequence and the amino acid at carboxyl terminus

Proteins were hydrolyzed in 5.7 N HCl in evacuated, sealed tubes for 24 and 72 hr at 110°C.   The amino acid composition of resulting hydrolysate was determined with automated amino acid analyzer model A3300 (Irica Instruments Inc., Kyoto, Japan).   The manual Edman degradation and identification of phenyl-thio-hydantoin (PTH) derivatives by thin layer chromatography were carried out as described by Hase et al. (1978). PTH derivatives were also identified by high performance liquid chromatography (Zimmerman et al., 1977).   Determination of the amino acid at carboxyl terminus was performed by the method of hydrazine degradation (Narita, 1970).

Primary structure of mutant DNA-binding protein

Wild-type T7 DNA-binding protein is consisted of 231 amino acid residues and has a calculated molecular weight of 25,562 (Dunn and Studier, 1981). One of the characteristics of this protein is that there is a cluster of the acidic amino acids in the carboxyl terminal region (Fig. IV-1b).

T7UP-2 strain seems to have a nonsense mutation in the carboxyl terminal region of T7 DNA-binding protein, since a UP-2 strain synthesized a 10% smaller protein in a molecular weight than wild-type T7 DNA-binding protein and synthesized normal size protein in a suppressive condition (Araki and Ogawa, 1981b; Chapter III). Therefore, the nucleotide sequence around the carboxyl terminal region was determined to find the mutation site of UP-2 strain. Because the gene 2.5 is translated from nucleotide 9,158 to nucleotide 9,853 (Dunn and Studier, 1981), the nucleotide sequence was determined in r strand from HinfI site at nucleotide 9,824 to nucleotide 9,769 and in l strand from AluI site at nucleotide 9,802 to nucleotide 9,861 using the specific fragments isolated from T7UP-2 phage DNA as described in MATERIALS AND METHODS. The nucleotide sequence revealed that a UP-2 mutation is resulted from TGG to TGA change on l strand at nucleotide 9,805. This result indicates that a UP-2 mutation is an opal mutation. The nucleotide sequence of a UP-2 mutant DNA also predicted that mutant protein is consisted of 214 amino acid residues and has a calculated molecular weight 23,570. From this prediction mutant protein lacks carboxyl terminal 17 amino acids of wild-type protein, containing 6 asparatic acids, 6 glutamic acids, and one of each of serine, glycine, alanine and phenyl-
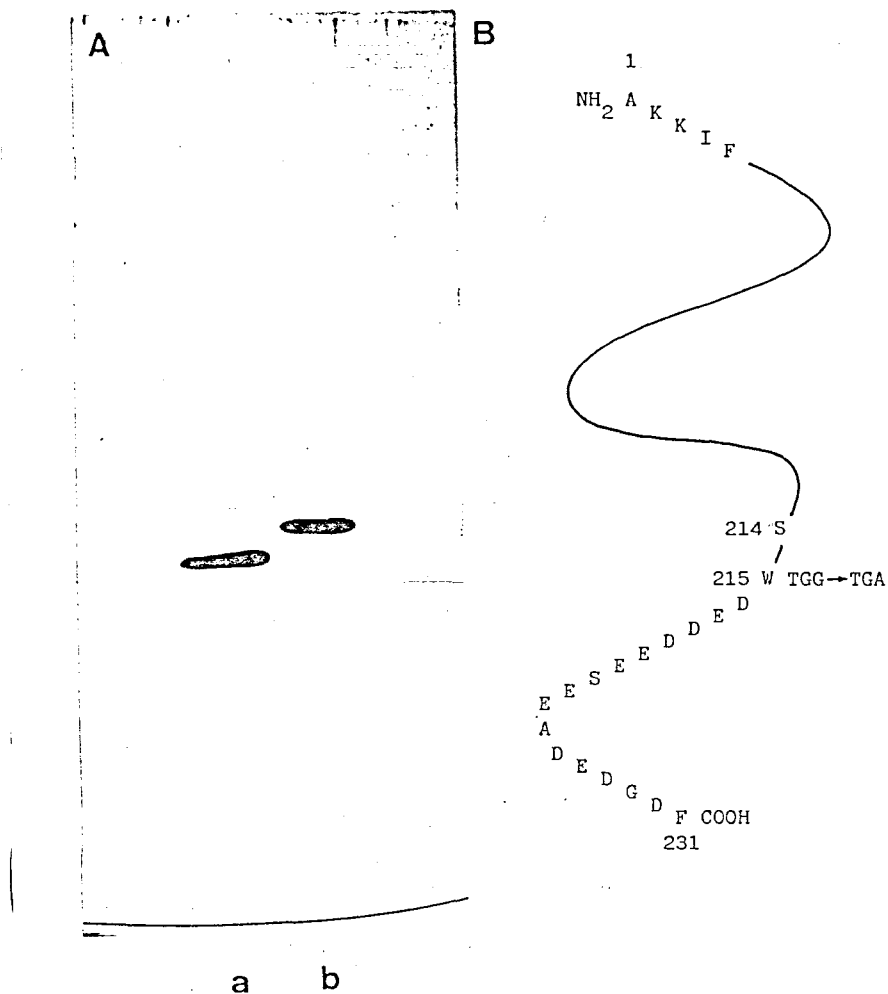
Figure IV-1.

 A. Purified wild-type (b) and mutant (a) proteins were subjected to 12.5%
SDS-polyacrylamide gel electrophoresis after heat denaturation of proteins.
The gel was stained with coomassie brilliant blue G-250.   The origin
of electrophoresis is at the top in the figure.

 B. A schematic representation of primary structure of T7 DNA-binding
protein.   A mutant protein consists of 214 amino acids in the result
of a change of tryptophan codon TGG to TGA at amino acid 215.

alanine, and the amino acid at carboxyl terminus of mutant protein is a
serine.

Mutant DNA-binding protein and wild-type DNA-binding protein were
purified by examining its amount with SDS-polyacrylamide gel electrophoresis
as described in MATERIALS AND METHODS. Both purities in mutant and
wild-type protein analyzed by SDS-polyacrylamide gel electrophoresis are
more than 90% (Fig. IV-1a). To identify the purified protein as mutant
DNA-binding protein, its amino acid composition and amino terminal amino
acid sequence were determined. There is excellent agreement between the
measured amino acid composition and predicted composition from the DNA
sequence (Table IV-1). The sequence found for the first five amino
acid residues at the amino terminus was exactly same as that of wild-
type protein, that is, NH_2-Ala-Lys-Lys-Ile-Phe. Therefore, the purified
protein should be mutant T7 DNA-binding protein synthesized by a UP-2
mutant. Comparison of the amino acid composition between mutant and
wild-type protein shows the decrease of the content of glutamic acid and
asparatic acid in mutant protein, meaning that acidic carboxyl terminal
region is deleted by the result of the nonsense mutation. The hydrazine
degradation of 5 nmols mutant protein released 3.2 nmols of serine, 1.4
nmols of glycine, 0.4 nmols each of asparatic acid and glutamic acid.
Since a serine is recovered as a major product of the hydrazine degradation
except hydrazides, mutant protein seems to have a serine at carboxyl
terminus as predicted from nucleotide sequence. Thus purified mutant
DNA-binding protein is the intact product without degradation by its
unstability or protease susceptability. The isoelectric point of
mutant protein in the presence of urea is 5.4 in contrast with 4.7 of
that of wild-type protein. It is consistent with the removal of acidic
amino acids. These results suggest that mutant DNA-binding protein is

Table IV-1

COMPARISON OF AMINO ACID COMPOSITION OF A MUTANT PROTEIN
WITH THAT OF A WILD-TYPE PROTEIN

Amino acid	Wild		UP-2	
	Predicted	Analysis	Predicted	Analysis
Asx	28	29.2	22	23.4
Thr	10	8.3	10	9.8
Ser	12	9.3	11	10.7
Glx	29	31.5	23	25.5
Pro	13	11.7	13	13.2
Gly	20	22.5	19	18.2
Ala	21	23.0	20	19.3
Val	18	21.1	18	19.5
Met	4	3.1	4	3.5
Ile	7	6.5	7	5.8
Leu	10	10.1	10	10.7
Tyr	11	8.7	11	9.1
Phe	9	8.6	8	8.1
Lys	25	23.8	25	23.4
His	2	1.9	2	2.0
Arg	6	5.6	6	6.7
Total	225	224.9	209	208.6

consisted of the amino acid terminal 214 amino acid residues of wild-type
protein (Fig. IV-1b).

DNA binding activity

   T7 DNA-binding protein binds strongly to single-stranded DNA without
Mg^{2+} by measuring sucrose gradient centrifugation.   When ^{3}H-labelled
Φx174 phage DNA (1 μg/ml) was incubated with T7 DNA-binding protein in the
absence of Mg^{2+}, DNA-protein complex was not retained on nitrocellulose
filter (less than 1%).   If Mg^{2+} was added to a reaction mixture, DNA was
retained.   This evidence indicates that T7 DNA-binding protein requires
Mg^{2+} for binding to nitrocellulose.   So, the reaction mixture always
contained Mg^{2+}.   A plot of retained fraction of single-stranded DNA
against the concentration of wild-type DNA-binding protein is sigmoidal
whereas a same plot for mutant DNA-binding protein is linear (Fig. IV-2a).
Retained fraction of single-stranded DNA reached to 100% at 20 μg/ml
wild-type protein.   At low concentration (0.1 - 10 μg/ml) of DNA-binding
protein, more single-stranded DNA was retained with mutant protein than
wild-type protein.   That is, at low concentration, mutant protein binds
to single-stranded DNA more efficiently than wild-type protein does and
vice versa at high concentration.

   Double-stranded DNA was reatined on nitrocellulose filter after
incubation with mutant protein at the concentration, above 10 μg/ml,
while with wild-type protein at the concentration, above 100 μg/ml
(Fig. IV-2b).   Though the mutant protein can bind to double-stranded
DNA more efficiently than wild-type protein does, it still preferentially
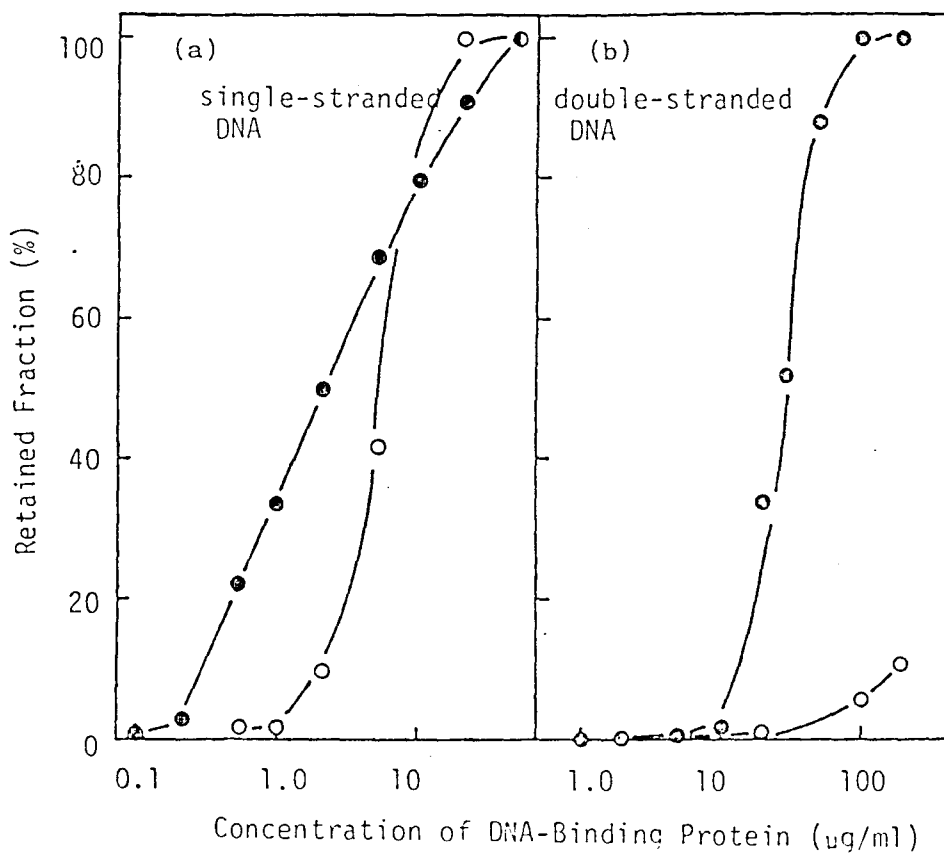binds to single-stranded DNA than double-stranded DNA.

Figure IV-2. DNA binding of mutant protein (●) and wild-type

protein (O). ^{3}H-labelled φx174 phage DNA (1 μg/ml) (a) or φx174 RFI

DNA (1 μg/ml) (b) was incubated at 30°C with mutant or wild-type

protein at indicated concentration for 10 min. After the sample was

passed through nitrocellulose filter, the radioactivity retained on
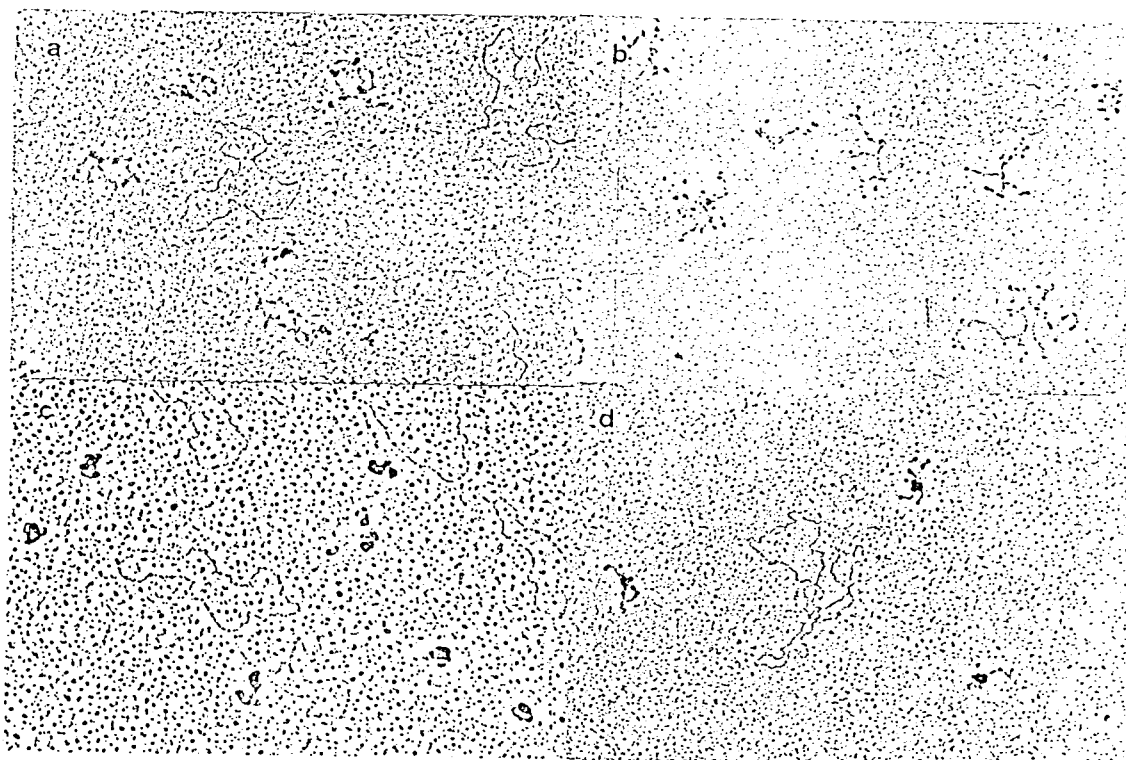
the filter was measured.

Figure IV-3.   Electron microscopy of complexes of φx174 DNA with a

mutant and a wild-type protein.   φx174 DNA (2 µg/ml) was incubated with

mutant (100 µg/ml) (b, d) or   wild-type protein (100 µg/ml) (a, c)

at 30°C in the presence (c, d) or absence (a, b) of Mg^{2+}.   The samples

were fixed with 0.01 M glutaraldehyde and spread with the protein free

φx174 DNA.   Magnification x 27,500.

Binding complex of DNA-binding protein with single-stranded DNA was examined by electron microscopy. The complex of wild-type protein with single-stranded DNA in the absence of Mg^{2+} had a condensed appearance like a beaded necklace (Fig. IV-3a) as observed in T3 DNA-binding protein (Yamagishi et al., 1981). The complex of mutant protein with single-stranded DNA in the same condition had a more condensed form like knot-like structure (Fig. IV-3b). In the presence of Mg^{2+}, both complexes of wild-type and mutant protein with single-stranded DNA become more and more condensed form like a bead (Fig. IV-3c, 3d).

All these results suggest that mutant protein binds to both single-stranded and double-stranded DNA more efficiently than wild-type protein does.

Renaturation of homologous single-stranded DNA

T7 DNA-binding protein stimulates the renaturation of homologous single-stranded DNA (Sadowski et al., 1980), which might be involved in the pairing process of genetic recombination. To examine the effect of the UP-2 mutation on the renaturation activity of T7 DNA-binding protein, heat denatured ^{3}H-labelled T7 DNA (2 μg/ml) was incubated at 30°C for 1 hr with various amount of wild-type or mutant protein in the absence or the presence of Mg^{2+}. The amount of renaturation of single-stranded DNA was deduced from measuring the amount of the fraction resistant to S1 nuclease.

In the absence of Mg^{2+}, S1 nuclease-resistant fraction increased steeply with the concentration of wild-type protein from 5 μg/ml to 30 μg/ml and had a maximal value, 80%, at 30 μg/ml (Fig. IV-4a). At higher concentrations of wild-type protein, a rapid decrease in S1-resistant
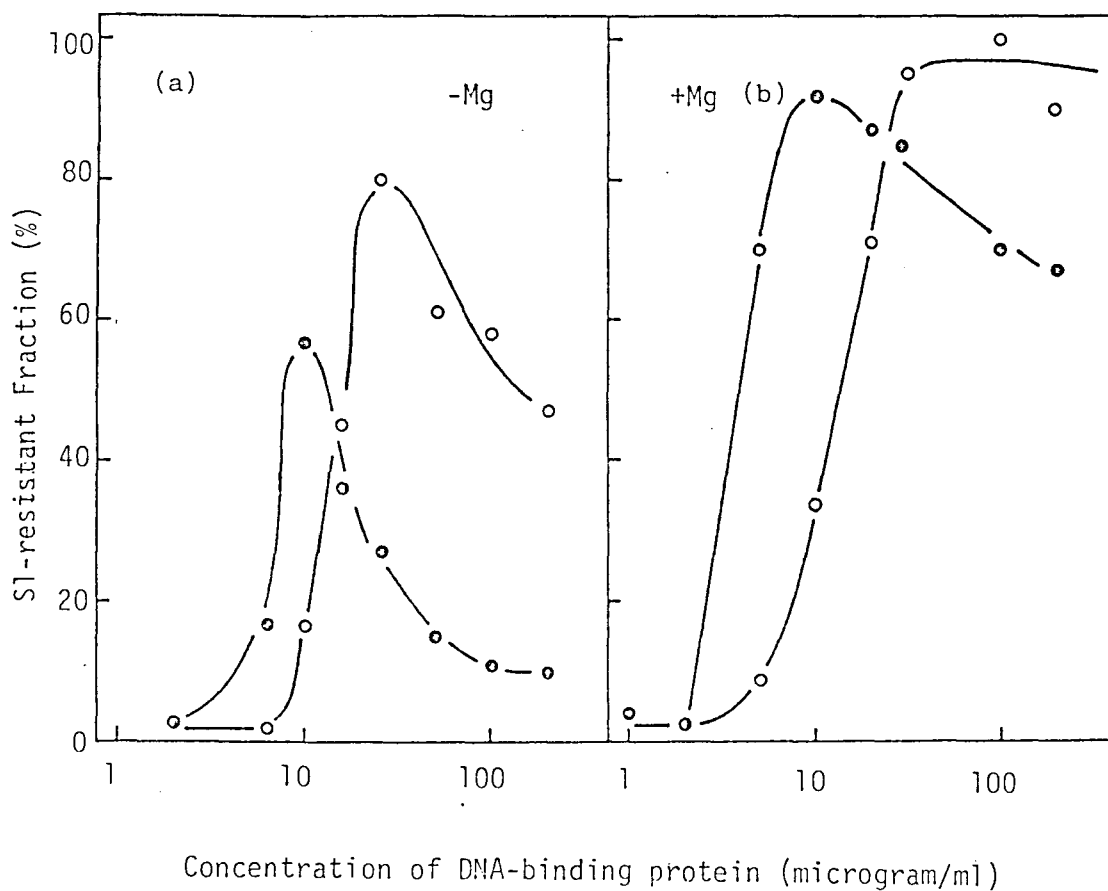
Figure IV-4.   Renaturation activity of   mutant (●) and   wild-type (○) protein in the absence (a) and the presence (b) of Mg^{2+}.

Heat-denatured ^3H-labelled T7 DNA (2 μg/ml) was incubated at $30°C$ with   mutant and   wild-type protein at indicated concentration for 1 hr. After addition of 1/10 volume of 10% SDS, the sample was treated with S1 nuclease and the acid-insoluble fraction was measured.
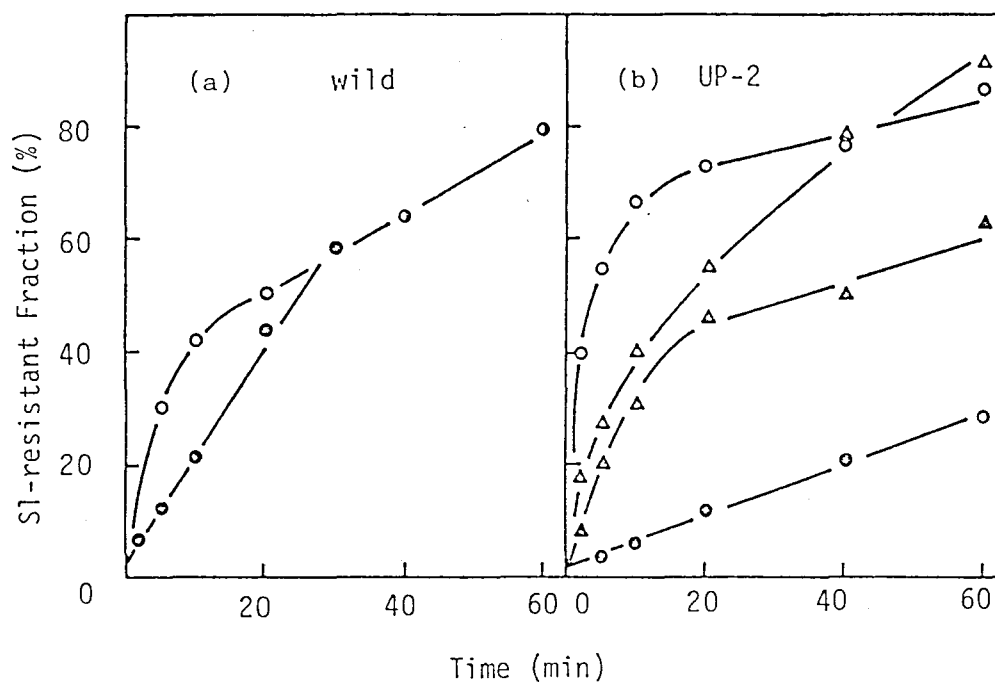
Figure IV-5.   Time course of renaturation.

a. Heat-denatured ^{3}H-labelled T7 DNA (2 µg/ml) was incubated at 30°C with

25 µg/ml of   wild-type protein for indicated times in the presence (O) or

the absence (◓) of Mg^{2+}.

b. Heat-denatured ^{3}H-labelled T7 DNA (2 µg/ml) was incubated at 30°C with

10 µg/ml (△▲) or 25 µg/ml (O◓) of   mutant protein for indicated times

in the presence (△O) or the absence (▲◓) of Mg^{2+}.

fraction was observed.   In the case of mutant protein, similar curve

was obtained but the maximal value, 60%, was attained at .   lower protein

concentration (10 μg/ml) than wild-type protein (30 μg/ml).

In the presence of Mg^{2+}, S1-resistant fraction reached to the maximal

value at the same protein concentration as in the absence of Mg^{2+}, for

both proteins, but the maximal values were   . higher (Fig. IV-4b).

Moreover, S1-resistant fraction scarcely decreased at higher concentrations

of wild-type protein but decreased significantly at those of mutant protein.

This result indicates that Mg^{2+} ions stimulate renaturation catalyzed by

T7 DNA-binding protein.   Both in the absence and in the presence of

Mg^{2+}, at low concentration of T7 DNA-binding protein, mutant protein is

also more efficient in renaturation than wild-type protein does and vice

versa at high concentration.   However, at the optimal condition for

wild-type protein, mutant protein showed less efficient renaturation activity.

Kinetics of renaturation was also examined for both wild-type and

mutant protein (Fig. IV-5).   For wild-type protein, S1-resistant fraction

was increased steeply for 10 min and then gradually for further 50 min, in

the presence of Mg^{2+}.   However, in the absence of Mg^{2+}, the steep increase

of S1-resistant fraction was not observed but   ·· slower increase

continued for 30 min.   For the mutant protein, at the same concentration

(25 μg/ml), S1-resistant fraction increased more steeply than wild-type

protein to a higher value for 10 min in the presence of Mg^{2+} However, in the

absence of Mg^{2+} no steep increase but far less gradual increase was observed.

At lower concentration (10 μg/ml), a rapid increase appeared again with

mutant protein even in the absence of Mg^{2+}.

These results revealed that although mutant protein holds a renaturation

activity, its activity is much more affected by its own concentration than

that of wild-type protein.   The relationship between the activity of

mutant protein and the lack of a joint molecules in mutant phage-infected cells is discussed later.

Denaturation activity of double-stranded DNA

Single-stranded DNA-binding proteins of bacteriophage T4 and E. coli lower the melting temperature of double-stranded DNA (Alberts and Frey, 1970; Sigal et al., 1972). But they cannot denature native double-stranded DNA completely. Proteolytic removal of carboxyl terminal region of T4 DNA-binding protein circumvents "the kinetic block" that may account for the failure to denature native double-stranded DNA (Jensen et al., 1976).

T7 DNA-binding protein also lowers the melting temperature of double-stranded DNA (Scherzinger et al., 1973). Denaturation activities of wild-type and mutant protein were compared. ^{3}H-labelled native double-stranded T7 DNA (2 µg/ml) was incubated at 30°C with 100 µg/ml of DNA-binding protein for 4 hr and S1-sensitive fraction was measured. Wild-type protein could not render double-stranded DNA sensitive to S1 nuclease either in the absence or presence of Mg^{2+}. But mutant protein made 10% of input double-stranded DNA sensitive to S1 nuclease in the absence of Mg^{2+} but not in the presence of Mg^{2+}. This result indicates that like T4 DNA-binding protein, removal of carboxyl terminal region of T7 DNA-binding protein makes it possible to denature native double-stranded DNA. It is also suggested that low renaturation activity of mutant protein in the absence of Mg^{2+} may be caused by its denaturation activity.

Stimulation of T7 exonuclease activity

T7 DNA-binding protein plays a role in the pairing process of genetic recombination in cooperation with T7 exonuclease in in vitro system
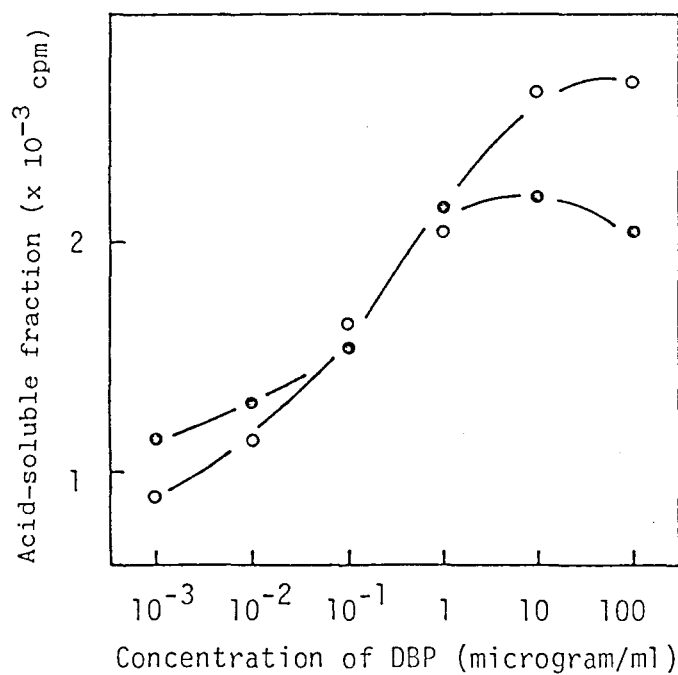
Figure IV-6.   Stimulation of T7 exonuclease activity by mutant (●) and wild-type (○) protein.   After ^{3}H-labelled T7 DNA (0.5 μg, 9,600 cpm) was

incubated at 30°C with 0.1 unit of T7 exonuclease and   mutant or

wild-type protein at indicated concentration for 30 min, the acid-

soluble fraction was measured.

(Sadowski et al., 1980; Araki and Ogawa, 1981a; Chapter II) and in

in vivo (Tsujimoto and Ogawa, 1977; Araki and Ogawa, 1981b; Chapter III).

It is also known that T7 DNA-binding protein stimulates the activity of

exonucleases including T7 exonuclease (Sadowski et al., 1980).   Thus

the effect of the mutant T7 DNA-binding protein on the stimulation of

T7 exonuclease activity was examined.

^{3}H-labelled T7 DNA (10 µg/ml) was incubated at 30°C with 0.1 unit of

T7 exonuclease and various amount of DNA-binding protein for 30 min.

As shown in Fig. IV-6, the activity of T7 exonuclease was increased with

the concentration of wild-type DNA-binding protein and levelled off at

10 µg/ml of DNA-binding protein.   But in the case of mutant protein,

the activity reached to the maximal level at 1 µg/ml of mutant DNA-

binding protein and the maximal level was lower than that of wild-type

protein.   At low concentrations of DNA-binding protein (10^{-3} and 10^{-2} µg/ml),

mutant protein stimulates the activity of T7 exonuclease more efficiently

than wild-type protein.   At high concentrations, however, wild-type

DNA-binding protein stimulates the activity of T7 exonuclease more efficiently

than mutant protein.

Stimulation of DNA synthesis

    T7 DNA-binding protein stimulates the activity of T7 DNA polymerase

(Reuben and Gefter, 1973, 1974; Scherzinger et al., 1973).   The effect

of the mutant protein on this stimulation activity was examined. T7 DNA

polymerase (0.24 unit) was incubated at 15°C with 5 µg/ml heat-denatured

T7 phage DNA and various amount of T7 DNA-binding protein for 30 min.-

As shown in Fig.IV-7a, in the case of wild-type protein, the incorporation

of ^{3}H-TMP increased with the concentration of DNA-binding protein and

levelled off at 25 µg/ml of DNA-binding protein.   The stimulation by

mutant protein increased with concentration until 25 μg/ml of DNA-binding

protein and decreased at higher concentrations.   The maximal level of

stimulation of mutant protein was lower than that of wild-type protein.

This result indicates that at low concentration, mutant protein stimulates

the activity of T7 DNA polymerase similarly to wild-type protein but

less efficiently than wild-type protein at higher concentration.

In the presence of four ribonucleotide triphosphates, T7 primase and

T7 DNA polymerase synthesize both the leading-strand

and lagging-strand using native double-stranded T7 phage DNA as a template.

T7 DNA-binding protein also stimulates this double-stranded DNA synthesis

(Scherzinger and Klotz, 1975; Richardson et al., 1978).   The effect of

the mutant protein on the double-stranded DNA synthesis was also examined.

T7 DNA polymerase (0.1 unit) and T7 primase (1.7 unit) were incubated at

30°C with 50 μM double-stranded T7 phage DNA and various amount of T7

DNA-binding protein   for 20 min.   As shown in Fig. IV-7b, mutant protein

could not stimulate DNA synthesis at all although wild-type protein

stimulated DNA synthesis in proportion to the concentration of DNA-binding

protein.   Therefore, mutant protein cannot work with T7 DNA polymerase

and T7 primase in the replication of double-stranded DNA.   It is

consistent with in vivo result described previously (Araki and Ogawa,
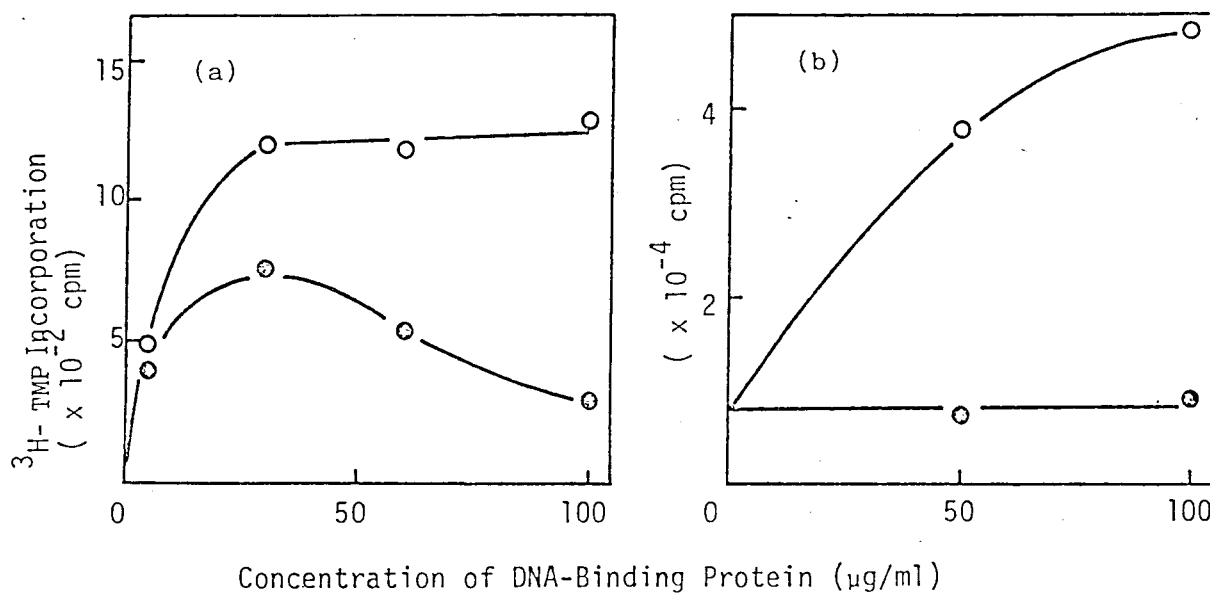
1981b; Chapter III).

Figure IV-7.   Stimulation of DNA synthesis by   mutant (●) and   wild-type (O) DNA-binding protein.

a. Heat-denatured T7 DNA (5 µg/ml) and 0.2 unit of T7 DNA polymerase were mixed and incubated at 15°C with   mutant or   wild-type protein at indicated concentration for 30 min.

b. Native double-stranded T7 DNA (5 nmol), 0.1 unit of T7 DNA polymerase and 1.7 unit of T7 primase were mixed and incubated at 30°C with indicated concentration of   mutant or   wild-type protein for 20 min.

Mutant protein stimulates the activity of T7 DNA polymerase to a single-stranded DNA template by different mode from wild-type protein, but cannot stimulate the replication of double-stranded DNA catalyzed by T7 DNA polymerase and T7 primase.

DISCUSSION

Physicochemical properties and DNA binding

Single-stranded DNA-binding proteins of E. coli and bacteriophage T4 have a homologous structure; the amino terminal portion has positive charges whereas the carboxyl terminal portion has negative charges (Sancar et al., 1981; Williams et al., 1980). The amino acid sequence predicted from the nucleotide sequence (Dunn and Studier, 1981) shows that the same is true for T7 DNA-binding protein .

Analyses of nucleotide sequence of the 2.5 gene of T7UP-2 and of the amino acid at carboxyl terminus of UP-2 mutant protein revealed that the mutant protein lacks 17 amino acids of carboxyl terminal portion of wild-type T7 DNA-binding protein, where 12 acidic amino acids clustered. Hence, the isoelectric point of the mutant protein increased in the result of deleting carboxyl terminal region. The mutant protein binds to single-stranded DNA more efficiently than does wild-type protein. Moreover, it can bind to double-stranded DNA in contrast with wild-type protein. In the case of T4 or E. coli DNA-binding protein, the proteolytically cleaved proteins which lost a native carboxyl terminal region show similar properties to the T7 mutant protein. They bind more efficiently than do intact proteins to single-stranded DNA and become to be able to bind to double-stranded DNA (Williams et al., 1981; Newport et al., 1980; Burke et al., 1980; Moise and Hosoda, 1976). Thus they may have a similar regulation system of DNA binding by its carboxyl terminal region.

Although the UP-2 mutation is recessive to the wild-type, the mutant protein seems to behave dominant against the wild-type protein in in vitro properties. This dominant properties of the mutant protein might be

reduced in the mutant phage infected cells by interaction with bacterial

DNA–binding protein.

Functions involved in genetic recombination and replication

T7UP-2 phage lacks the abilities for DNA replication and for the

formation of recombinational intermediates.   To form recombinational

intermediates both T7 exonuclease and T7 DNA-binding protein were required

(Tsujimoto and Ogawa, 1977; Sadowski et al., 1980; Araki and Ogawa, 1981a;

Chapter II; Chapter III).   The functions of T7 DNA-binding protein

involved in genetic recombination may be its renaturation activity and or

its stimulation activity for T7 exonuclease.   The mutant DNA-binding

protein has much less renaturation activity and stimulation activity for

the exonuclease at the   concentration optimal

for wild-type protein, although at very low concentration, both activities

of the mutant protein are more efficient   than wild-type protein.

In the infected cells, both less efficient activities of the mutant protein

may additively affect the formation of recombinational intermediates,

and hence lower the recombination frequency in a physiological condition.

Mutant DNA-binding protein stimulates T7 DNA polymerase activity for

single-stranded DNA template at different mode from wild-type protein:

The stimulation increased with concentration of DNA-binding protein at

low concentrations (less than 25 µg/ml) but decreased at higher

concentrations (more than 25 µg/ml), although the stimulation by wild-

type protein increased and then levelled off with the concentration added.

A maximal level of stimulation of mutant protein is lower than that of

wild-type protein.   A decrease of stimulation of mutant protein at

higher concentration is similar to the effect of T4 32*I protein on

T4 DNA polymerase since T4 32*I protein inhibits T4 DNA polymerase activity

for a nicked double-stranded DNA template and preprimed single-stranded

DNA template (Burke et al., 1980).   Moreover, mutant protein cannot

stimulate the replication of double-stranded DNA catalyzed by T7 DNA

polymerase and T7 primase.   Similar effect was also observed in T4

32*I protein and was resulted from the reduction of RNA priming and

primer utilization (Burke et al., 1980).   T4 32*I protein cannot

interact with T4 DNA polymerase and one of T4 RNA priming proteins

(Burke et al., 1980).   From the analogy of T4 DNA-binding protein

the interactions of T7 DNA-binding protein with T7 replication enzymes

and with template DNA should be important for DNA replication.

Role of carboxyl terminal region

     Mutant protein has the activity unwinding native double-stranded

T7 DNA as T4 32*I protein has.   Moise and Hosoda (1976) suggested

that the carboxyl terminal region of T4 DNA-binding protein plays

a role of controlling the unwinding activity and the interaction

with other T4 DNA replication proteins.   From the analogy lacking carboxyl

terminal region, it is likely that similar regulation is exsisted in

T7 DNA-binding protein.   Activity of DNA-binding protein in T7UP-2-

infected cells may be low by losing regulation of its activity in addition

to its own lower activity by the mutation.

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