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THE EARLY PROCESS OF GENETIC RECOMBINATION

——ROLE OF T7 DNA-BINDING PROTEIN——

By

HIROYUKI ARAKI

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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

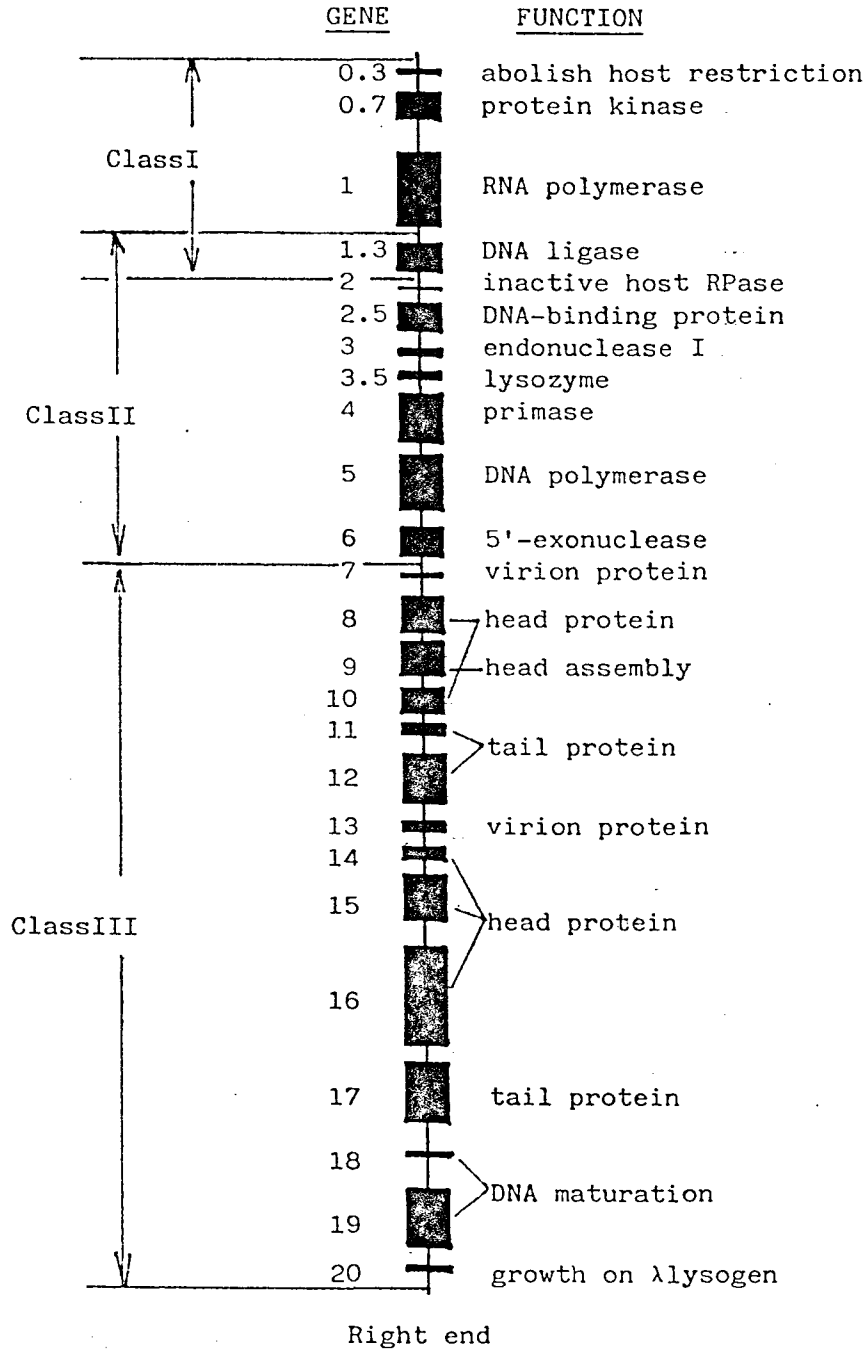

Figure I-1. Genetic map of phage T7

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 polymerase 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

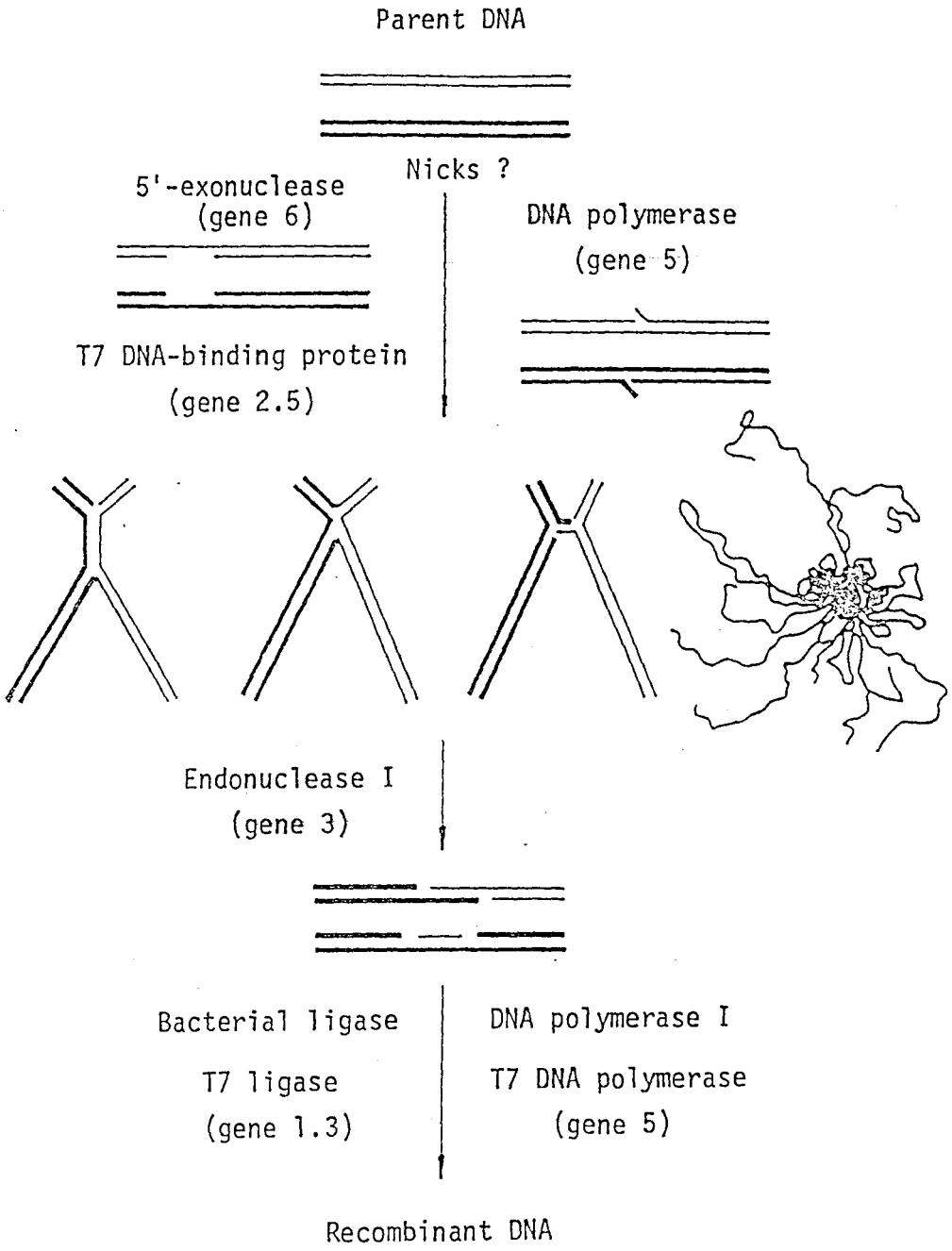

Figure I-2. Schematic representation of a process of genetic recombination in bacteriophage T7.

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 characterizing 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.

REFERENCES

Alberts, B. M. and Frey, L. (1970) T4 bacteriophage gene 32: A structural protein in the replication and recombination of DNA. Nature 227, 1313-1318.

Bernstein, C. (1981) Deoxyribonucleic acid repair in bacteriophage. Microbiol. Rev. 45, 72-98.

Broker, T. R. and Lehman, I. R. (1971) Branched DNA molecules: Intermediates in T4 recombination. J. Mol. Biol. 60, 131-149.

Center, M. S. (1975) Role of gene 2 in bacteriophage T7 DNA synthesis. J. Virol. 16, 94-100.

Champoux, J. J. (1978) Proteins that affect DNA conformation. Annu. Rev. Biochem. 47, 449-480.

DeWyngaert, M. A. and Hinkle D. C. (1979) Bacterial mutants affecting phage T7 DNA replication produce RNA polymerase resistant to inhibition by the T7 gene 2 protein. J. Biol. Chem. 254, 11247-11253.

Dunn, J. J. and Studier, F. W. (1981) Nucleotide sequence from the genetic left end of bacteriophage T7 DNA to the beginning of gene 4. J. Mol. Biol. 148, 303-330.

Epstein, R. H., A. Bolle, A., Steinberg, C. M., Kellenberger, E., Boy de la Tour, E., Chevalley, R., Edgar, R. S., Susman, M., Denhardt, G. H., and Lielausis, A. (1963) Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. 28, 375-394.

Fischer, H. and Hinkle, D. C. (1980) Bacteriophage T7 DNA replication in vitro. J. Biol. Chem. 255, 7956-7964.

Glassberg, J., Meyer, R. R. and Kornberg, A. (1979) Mutant single-strand binding protein of Escherichia coli: Genetic and physiological

characterization. J. Bacteriol. 140, 14-19.

Hausmann, R. (1976) Bacteriophage T7 genetics. Current. Topics Microb. Immunol. 75, 77-110.

Hinkle, D. C. (1980) Evidence for direct involvement of T7 RNA polymerase in bacteriophage DNA replication. J. Virol. 34, 136-141.

Horii, Z. I. and Clark, A. L. (1973) Genetic analysis of the recF pathway to genetic recombination in Escherichia coli K12: Isolation and characterization of mutans. J. Mol. Biol. 80, 327-344.

Johnson, B. F. (1977) Genetic mapping of the lexC-113 mutation. Mol. Gen. Genet. 157, 91-97.

Kerr, C. and Sadowski, P. D. (1975) The involvement of genes 3, 4, 5 and 6 in genetic recombination in bacteriophage T7. Virology 65, 281-285.

Kolodner, R. and Richardson, C. C. (1977) Replication of duplex DNA by bacteriophage T7 DNA polymerase and gene 4 protein is accompanied by hydrolysis of nucleoside 5'-triphosphates. Proc. Natl. Acad. Sci. USA 74, 1527-1529.

MacKay, V. and Linn, S. (1974) The mechanism of degradation of duplex deoxyribonucleic acid by the recBC enzyme of Escherichia coli K12. J. Biol. Chem. 249, 4286-4294.

MacKay, V. and Linn, S. (1976) Selective inhibition of the DNase activity of the recBC enzyme by the DNA-binding protein from Escherichia coli. J. Biol. Chem. 251, 3716-3719.

Masamune, Y., Frenkel, G. D. and Richardson, C. C. (1971) A mutant of bacteriophage T7 deficient in polynucleotide ligase. J. Biol. Chem. 246, 6874-6879.

McEntee, K. Weinstock, G. M. and Lehman, I. R. (1980) RecA protein-catalyzed strand assimilation: Stimulation by Escherichia coli single-stranded

DNA-binding protein. Proc. Natl. Acad. Sci. USA 77, 857-861.

Meyer, R. R., Glassberg, J. and Kornberg, A. (1979) An Escherichia coli mutant defective in single-strand binding protein is defective in DNA replication. Proc. Natl. Acad. Sci. USA 76, 1702-1705.

Moise, H. and Hosoda, J. (1976) T4 gene 32 protein model for control of activity at replication fork. Nature 259, 455-458.

Molineux, I. J. and Gefter, M.L.(1974) Properties of the Escherichia coli DNA binding (unwinding) protein: Interaction with DNA polymerase and DNA. Proc. Natl. Acad. Sci. USA 71, 3858-3862.

Molineux, I. J. Gefter, M. L. (1975) Properties of the Escherichia coli DNA-binding (unwinding) protein interaction with nucleotic enzymes and DNA. J. Mol. Biol. 98, 811-825.

Mosig, G., Luder, A., Garcia, G., Dannenberg, R. and Bock, S. (1978) In vivo interactions of genes and proteins in DNA replication and recombination of phage T4. Cold Spring Harbor Symp. Quant. Biol. 43, 501-515.

Ogawa, H., Araki, H. and Tsujimoto , Y. (1978) Recombination intermediates formed in the extract from T7-infected cells. Cold Spring Harbor Symp. Quant. Biol. 43, 1033-1041.

Powling, A. and Knippers, R. (1974) Some functions involved in bacteriophage T7 genetic recombination. Mol. Gen. Genet. 134, 173-180.

Reuben, C. R. and Gefter, M. L. (1973) A DNA binding protein induced by bacteriophage T7. Proc. Natl. Acad. Sci. USA 70, 1846-1850.

Reuben, C. R. and Gefter, M. L. (1974) A deoxyribonucleic acid-binding protein induced by bacteriophage T7. Purification and properties of the protein. J. Biol. Chem. 249, 3843-3850.

Richardson, C. C., Romano, L. J., Kolodner, R., LeClerc, J. E., Tamanoi, F., Engler, M. J., Dean, F. B. and Richardson, D. S. (1978) Replication of

bacteriophage T7 DNA by purified proteins. Cold Spring Harbor Symp. Quant. Biol. 43, 427-440.

Sadowski, P. D. and Kerr, C. (1970) Degradation of Escherichia coli B deoxyribonucleic acid after infection with deoxyribonucleid acid-defective amber mutants of bacteriophage T7. J. Virol. 6, 149-159.

Sancar, A., Williams, K. R., Chase, J. W. and Rupp, W. D. (1981) Sequences of the ssb gene and protein. Proc. Natl. Acad. Sci. USA 78, 4272-4278.

Scherzinger, E., Litfin, F. and Jost, E. (1973) Stimulation of T7 DNA polymerase by a new phage-coded protein. Mol. Gen. Genet. 123, 247-262.

Scherzinger, E. and Klotz, G. (1975) Studies on bacteriophage T7 DNA synthesis in vitro. II. Reconstitution of the T7 replication system using purified proteins. Mol. Gen. Genet. 141, 233-249.

Scherzinger, E., Lanka, E., Morelli, G., Seiffert, D. and Yuki, A. (1977) Bacteriophage T7-induced DNA-priming protein. Eur. J. Biochem. 72, 543-558.

Shibata, T., DasGupta, C., Cunningham, R. P. and Radding, C. M. (1979) Purified Escherichia coli recA protein catalyzes homologous pairing of superhelical DNA and single-stranded fragments. Proc. Natl. Acad. Sci. USA 76, 1638-1642.

Shinozaki, K. and Okazaki, T. (1978) T7 gene 6 exonuclease has an RNase H activity. Nucl. Acids Res. 5, 4245-4261.

Sigal, N., Dēlius, H., Kornberg, T., Gēfter, M. L. and Alberts, B. (1972) A DNA-unwinding protein isolated from Escherichia coli: Its interaction with DNA and DNA polymerase. Proc. Natl. Acad. Sci. USA 69, 3537-3541.

Silberstein, S., Inouye, M. and Studier, F. W. (1975) Studies on the role of bacteriophage T7 lysozyme during phage infection. J. Mol. Biol.

96, 1-11.

Studier, F. W. (1969) The genetics and physiology of bacteriophage T7. Virology 39, 562-574.

Studier, F. W. (1972) Bacteriophage T7. Science 176, 367-376.

Tomizawa, J., Anraku, N. and Iwama, Y. (1966) Molecular mechanism of genetic recombination in bacteriophage. VI. A mutant defective in the joining of DNA molecules. J. Mol. Biol. 109, 423-436.

Tsujimoto, Y. and Ogawa, H. (1978). Intermediates in genetic recombination of bacteriophage T7 DNA. Biological activity and the roles of gene 3 and gene 5. J. Mol. Biol. 125, 255-273.

Williams, K. R., LoPresti, M. B., Setoguchi, M. and Konigsberg, W. H. (1980) Amino acid sequence of the T4 DNA helix-destabilizing protein. Proc. Natl. Acad. Sci. USA 77, 4614-4617.

Williams, K. R., Guggenheimer, R. A., Chase, J. W. and Konigsberg, W. H. (1981) Physicochemical properties of a limited proteolysis product of the E. coli single-stranded DNA binding protein (SSB). Fed. Proc. Fed. Am. Soc. Exp. Biol. 40, 1731.

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 ^3H-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°C in L-broth. After incubation at 37°C for 15 min, the infected cells were harvested by centrifugation at 0°C. 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°C, 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^2 x 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).

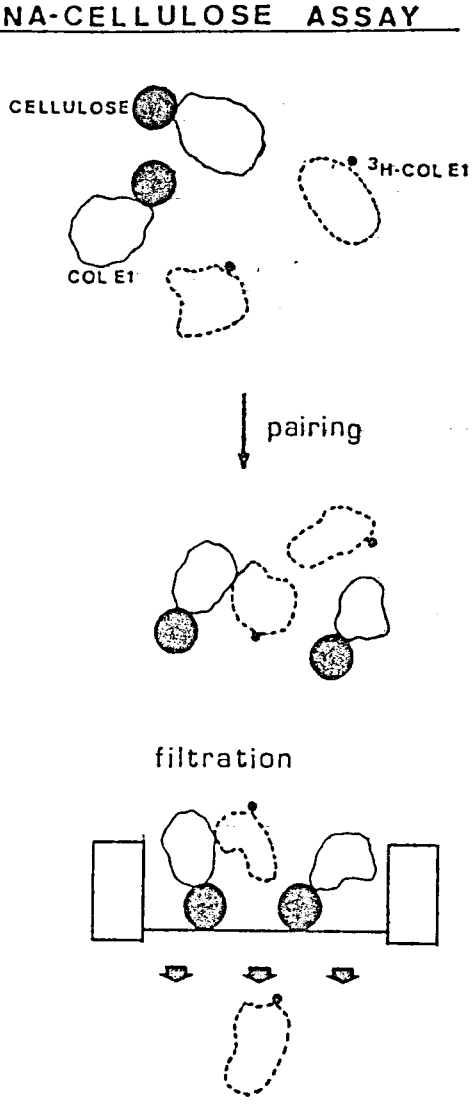

Figure II-1. A schematic representation of DNA-cellulose assay

See RESULTS for details.

RESULTS

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 cariied 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

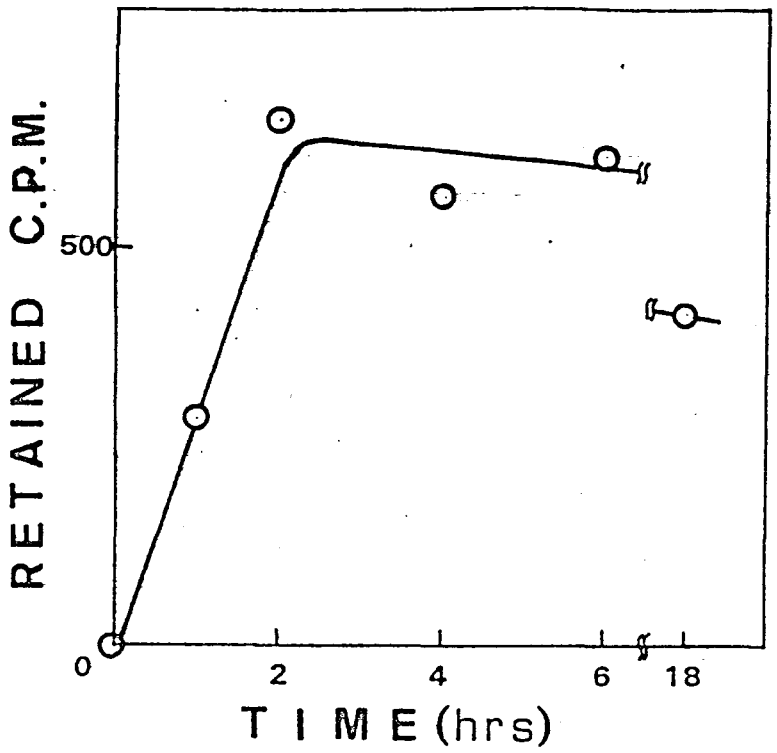

Figure II-2. A time course experiment on the radioactivity retained by DNA-cellulose in the extract. An aliquot of ColE1 DNA-cellulose powder (containing about 20 μg DNA) was added to 50 μl of a mixture composed of T7 2am 3am 4am 5am 6^+ - infected cells and 2 μg of ^{3}H-labelled ColE1 DNA (2×10^5 cpm). 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

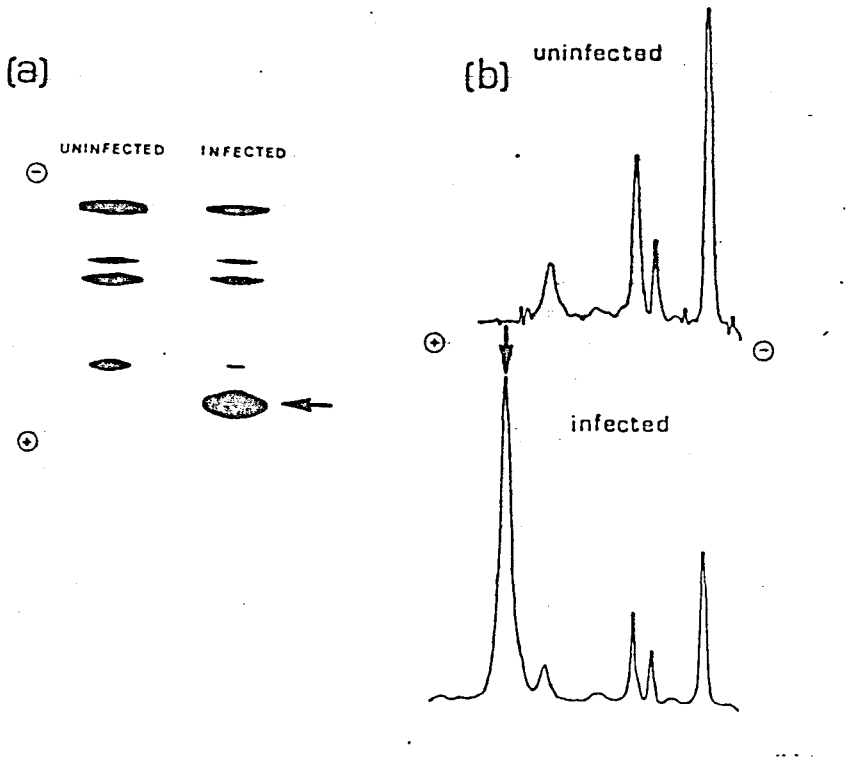

Figure II-3. Profiles of protein bands of 1 M eluate from DNA-agarose column on SDS-polyacrylamide gels and their scanned profiles.
(a) A photograph of protein bands stained on SDS-polyacrylamide gels. Left side; proteins from uninfected cells (5 μg). Right side; proteins from infected cells (5 μg). (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.

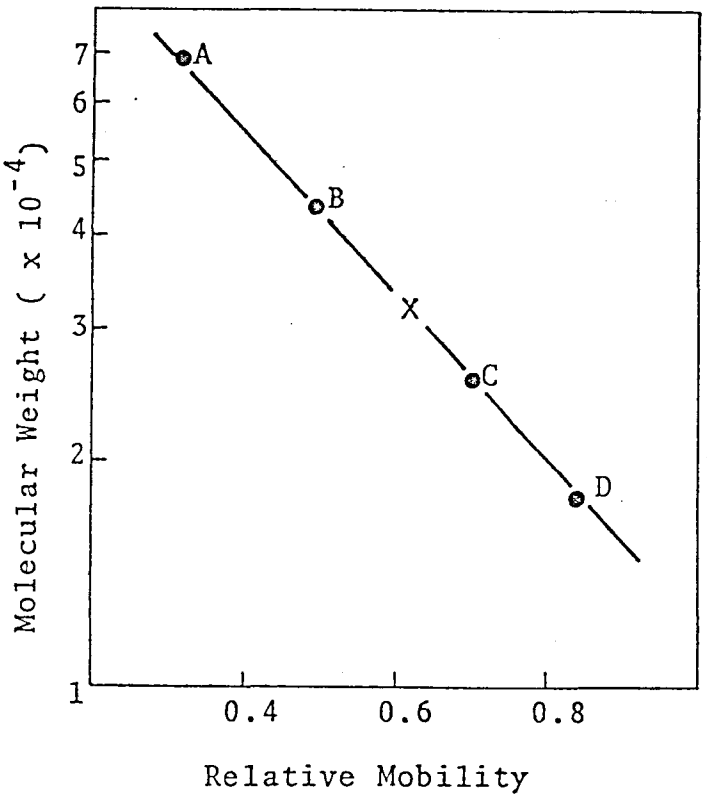

Figure II-4. Determination of molecular weight of characteristic purified protein extracted from infected cells by SDS-polyacrylamide gel electrophoresis. The method followed was generally as described in Weber and Osborn (1969). Protein standards were A: albumin (68,000 daltons) B; ovalbumin (43,000 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.

REFERENCES

Clewell, D. B. and Helinski, D. R. (1969). Supercoiled circular DNA-protein complex in Escherichia coli: Purification and induced conversion to an open-circular DNA form. Proc. Natl. Acad. Sci. USA 62, 1159-1166.

Hosoda, J. (1976). Role of gene 46 and gene 47 in bacteriophage T4 reproduction. III. Formation of joint molecules in biparental recombination. J. Mol. Biol. 106, 277-284.

Litman, R. M. (1968). A deoxyribonucleic acid polymerase from Micrococcus luteus(Micrococcus lysodeikticus) isolated on deoxyribonucleic acid-cellulose. J. Biol. Chem. 243, 6222-6233.

Ogawa, H., Araki, H. and Tsujimoto, Y. (1978). Recombinaiton intermediates formed in the extract from T7-infected cells. Cold Spring Harbor Symp. Quant. Biol. 43, 1033-1041.

Reuben, C. R. and Gefter, M. L. (1973). A DNA-binding protein induced by bacteriophage T7. Proc. Natl. Acad. Sci. USA 70, 1846-1850.

Reuben, C. R. and Gefter, M. L. (1974). A deoxyribonucleic acid-binding protein induced by bacteriophage T7. Purification and properties of the protein. J. Biol. Chem. 249, 3843-3850.

Sadowski, P. D. and Vetter, D. (1976). Genetic recombination of bacteriophage T7 DNA in vitro. Proc. Natl. Acad. Sci. USA 73, 692-696.

Sakakibara, Y. and Tomizawa, J. (1974). Replication of colicin E1 plasmid DNA in cell extracts. Proc. Natl. Acad. Sci. USA 71, 802-806.

Shaller, H., Nüsslein, C., Bonhoeffer, F. J., Kurtz, C. and Nietzschmann,I. (1972). Affinity chromatography of DNA-binding enzymes on single-

stranded DNA-agarose columns. Eur. J. Biochem. 26, 474-481.

Shapiro, A. L., Vinuela, E. and Maizel, J. V. (1967). Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. 28, 815-820.

Sherzinger, E. and Klotz, G. (1975). Studies of bacteriophage T7 DNA synthesis in vitro. II. Reconstitution of the T7 replication system using purified proteins. Mol. Gen. Genet. 141, 233-249.

Shinozaki, K. and Okazaki, T. (1978). T7 gene 6 exonuclease has an RNase H activity. Nucl. Acids Res. 5, 4245-4261.

Tomizawa, J., Anraku, N. and Iwama, Y. (1966). Molecular mechanism of genetic recombinaiton in bacteriophage. VI. A mutant defective in the joining of DNA molecules. J. Mol. Biol. 21, 247-253.

Tsujimoto, Y. and Ogawa, H. (1977). Intermediates in genetic recombination of bacteriophage T7 DNA. J. Mol. Biol. 109, 423-436.

Tsujimoto, Y. and Ogawa, H. (1978). Intermediates in genetic recombination of bacteriophage T7 DNA. Biological activity and the roles of gene 3 and gene 5. J. Mol. Biol. 125, 255-273.

Weber, K. and Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. 244, 4406-4412.

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 (2×10^8/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)
594*lexC113*	*sup*$^+$ *thyA deo*	PAM2611 X WD00, selection for *malB*$^+$ *lexC113*
594*ssb-1*	*sup*$^+$ *thyA deo*	P1(SG1635)→WD00, selection for *malB*$^+$ *ssb-1*
594*metEmalB*	*sup*$^+$ *thyA deo*	spontaneous *metE* mutant of WD00
594*trxAmalB*	*sup*$^+$ *thyA deo*	P1(JM110)→594*metEmalB*, selection for *metE*$^+$ *trxA*
594*trxAssb-1*	*sup*$^+$ *thyA deo*	P1(SG1635)→594*trxAmalB* 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)
C600*malE*	*glnU thyA deo thr leu*	obtained from Dr. Epstein
C600*lexC113*	*glnU thyA deo*	PAM2611 X C600, selection for *thr*$^+$ *leu*$^+$ *lexC113*
C600*ssb-1*	*glnU thyA deo thr leu*	P1(SG1635)→C600*malE*, selection for *malE*$^+$ *ssb-1*
JC1557	*sup59*	Clark *et al.* (1966)
JC1557*uvrA*	*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 x 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 x 10^9 infected cells.

Isolation of intermediate T7 DNA genetic recombinant molecules

Bacteria of 594trxAssb-1 were grown to 2 x 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°C for 5 min, and then treated with phage T7 specific antiserum at a final K value of 3 for 5 min at 37°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°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°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 C600*ssb-1* but not on 594*ssb-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 594*ssb-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 C600*ssb-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.

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 x 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 x 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.

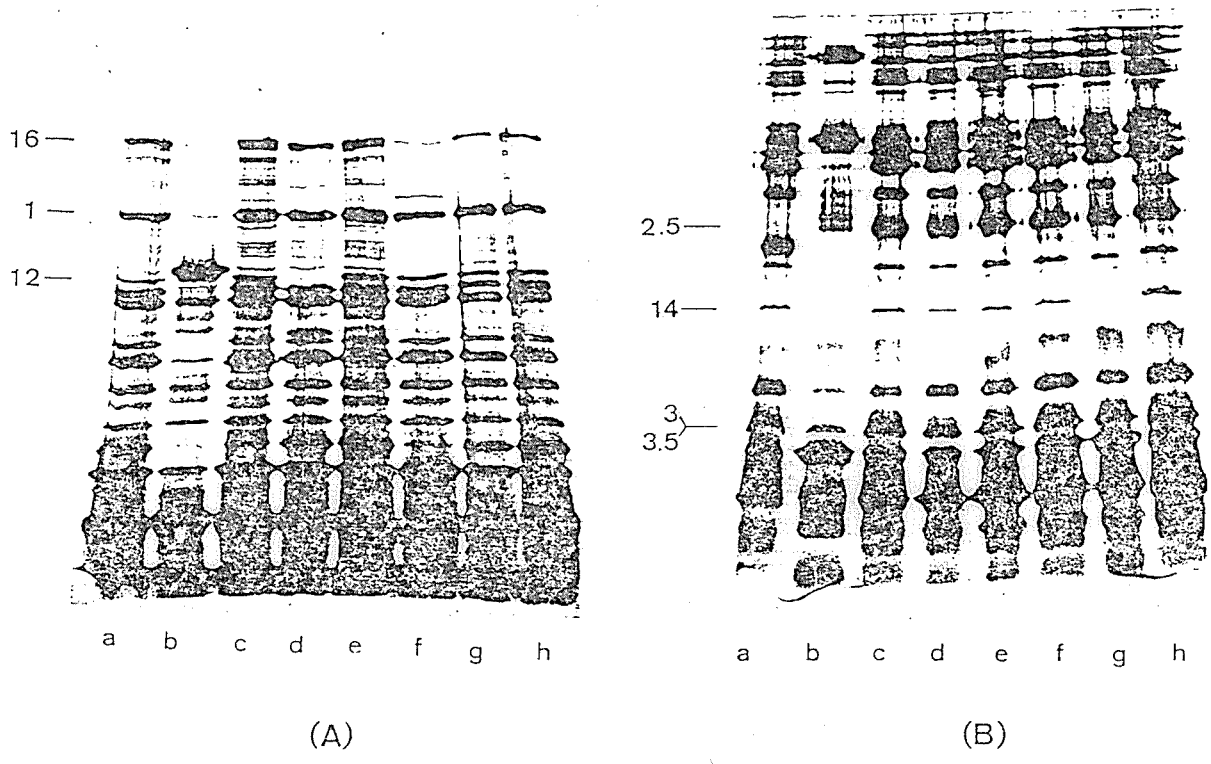

Figure III-1. Profiles of the proteins synthesized by various T7 phages on SDS-polyacrylamide gel electrophoresis.

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 electrophoresis 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.

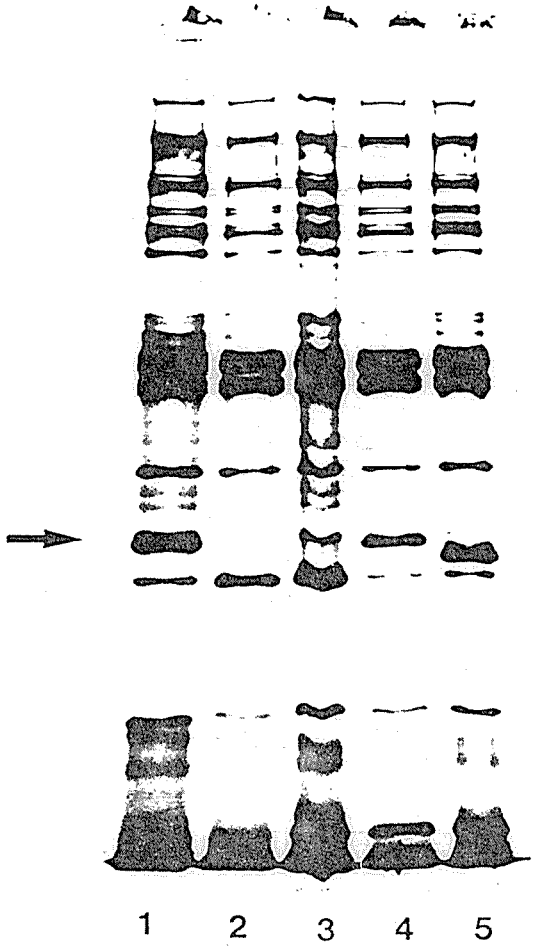

Figure III-2. Profiles of the proteins synthesized by various T7 phages on SDS-polyacrylamide gel electrophoresis.

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
C600*ssb-1*	0.29	0.10	0.86
C600*lexC113*	0.54	0.28	0.93
594	0.75	0.46	1.1
594*ssb-1*	0.23	1.6×10^{-5}	0.86
594*lexC113*	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.

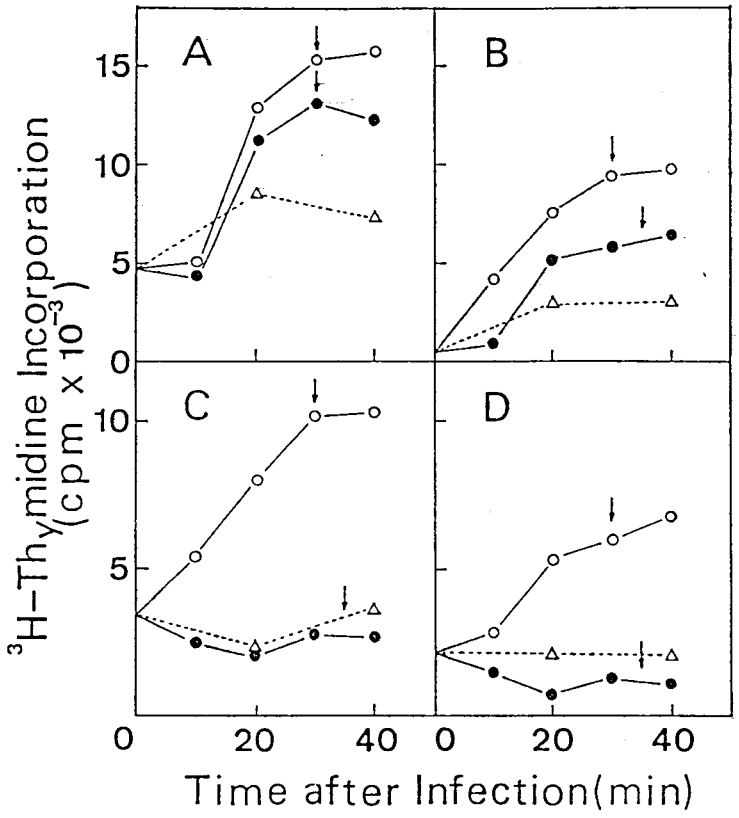

Figure III-3. Time course of DNA synthesis in the T7 infected cells.

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% trichloroacetic 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.

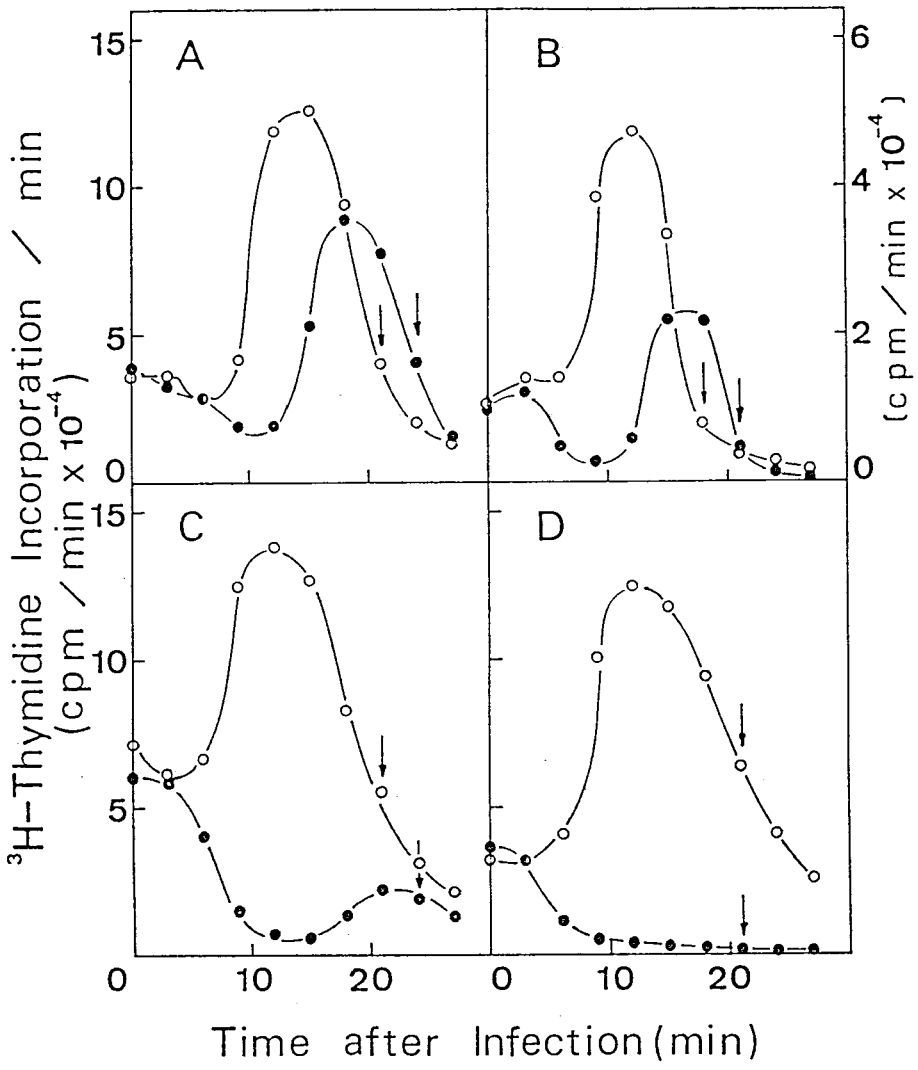

Figure III-4. Time course of the rate of the DNA synthesis after the infection of T7 phage.

Various cells, C600 (A), C600ssb-1 (B), WD00 (C) and 594ssb-1 (D), were infected with T7 wild-type (○) or UP-2 (●) at 37°C. A half milliter 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 WDOO 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 WDOO 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^{o}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

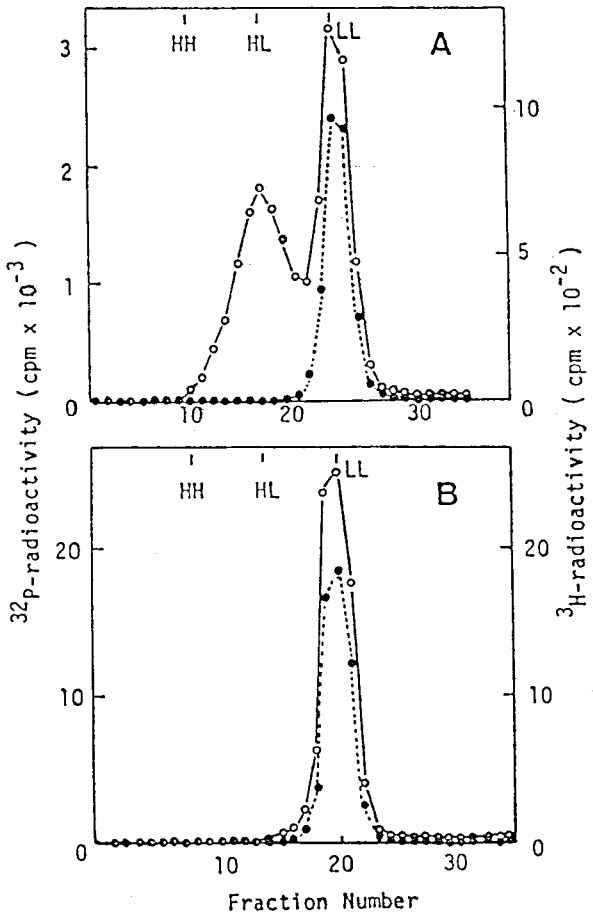

Figure III-5. Density labelling experiment of replication of T7 wild-type and T7 UP-2 mutant in 594ssb-1 host bacteria.

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 x UP-2am10	Q1	35	3.1
	594ssb-1	3	4.1
	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 2, 3, 4, and 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 (trx) which is a subunit of T7 DNA polymerase to prevent the synthesis of T7 DNA instead of using T7 mutants.

Bacteria, 594trxAssb-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.

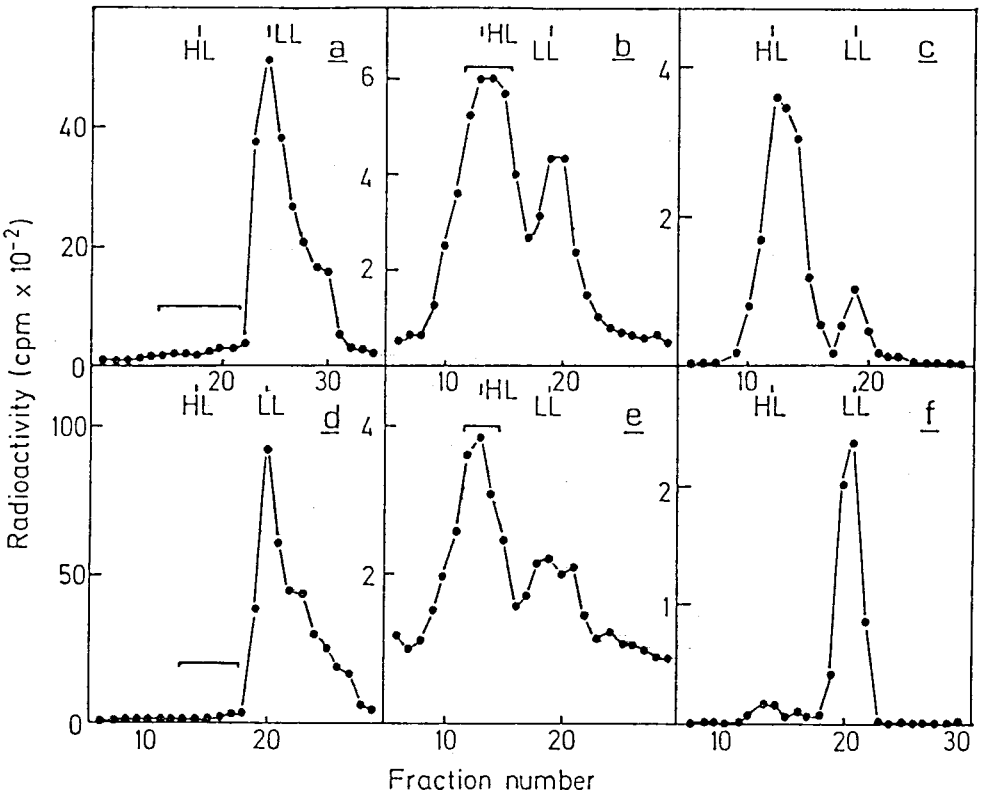

Figure III-6. Formation of intermediate density molecules in 594trxAssb-1 bacteria after infection with ^{32}P-labelled and BrdU-labelled UP-2 mutant phage.

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) Recentrifugation 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.

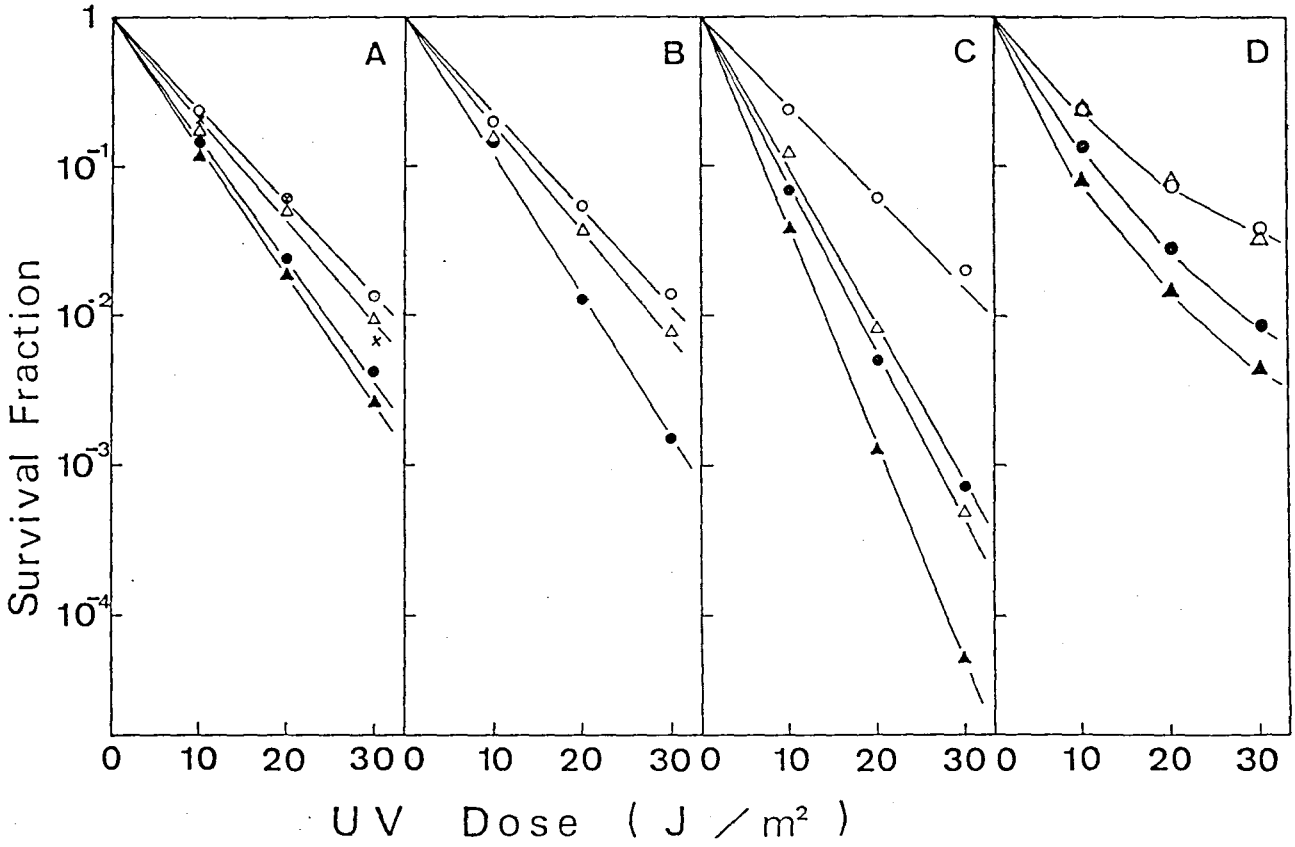

Figure III-7. UV sensitivity of T7 UP-2 phage

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 (○●×) and C600ssb-1(△▲) in (A), 594 (○●) and 594ssb-1 (△) in (B), JC1557 (○●) and JC1557uvrA (△▲) in (C), 594(pDR1996) (○●) and 594 (△▲) 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.

REFERENCES

Araki, H. and Ogawa, H. (1981). The participation of T7 DNA-binding protein in T7 genetic recombination. Virology 111, 509-515.

Baas, P. D., Keegstra, W., Teerstra, W. R. and Jansg, H. S. (1978) R-loops in bacteriophage ϕX174 RF DNA. J. Mol. Biol. 125, 187-205.

Campbell, A. (1965). The steric effect in lysogenization by bacteriophage lambda. I. Lysogenization of a partially diploid strain of Escherichia coli K12. Virology 27, 329-339.

Clark, A. J., Chamberlin, M., Boyce, R. P. and Howard-Flanders, P. (1966). Abnormal metabolic response to ultraviolet light of a recombination deficient mutant of Escherichia coli K12. J. Mol. Biol. 19, 442-454.

Clowes, R. C. and Hayes, W. (1968). Experiments in microbial genetics. Wiley, New York.

Dunn, J.J. and Studier, F. W. (1981). Nucleotide sequence from the genetic left end of bacteriophage T7 DNA to the beginning of gene 4. J. Mol. Biol. 148, 303-330.

Glassberg, J., Meyer, R. R. and Kornberg, A. (1979). Mutant single-strand binding protein of Escherichia coli: genetic and physiological characterization. J. Bacteriol. 140, 14-19.

Hausmann, R. (1976). Bacteriophage T7 genetics. Curr. Top. Microbiol. Immunol. 75, 77-110.

Ikeda, H. and Tomizawa, J. (1965). Transducing fragments in generalized transduction by phage P1. I. Molecular origin of the fragments. J. Mol. Biol. 14, 85-109.

Mark, F. D., Chase, J. M. and Richardson, C. C. (1977). Genetic mapping of trxA, a gene affecting thioredoxin in Escherichia coli K12. Mol. Gen. Genet. 155, 145-152.

Masukata, H., Ogawa, T. and Ogawa, H. (1979). The R-loop structure of λDNA in dnaG mutant of E. coli. Abstracts of the annual meeting of the American Society for Microbiology - 1979. 137.

Mayer, R. R., Glassberg, J. and Kornberg, A. (1979). An Escherichia coli mutant defective in single-strand binding protein is defective in DNA replication. Proc. Natl. Acad. Sci. USA 76, 1702-1705.

North, R. and Molineux, I. J. (1980). A novel mutant of bacteriophage T7 that is defective in early phage DNA synthesis. Mol. Gen. Genet. 179, 683-691.

Ogawa, T. and Tomizawa, J. (1967). Abortive lysogenization of bacteriophage lambda b2 and residual immunity of non-lysogenic segregants. J. Mol. Biol. 23, 225-245.

Ogawa, T. (1975). Analysis of the dnaB function of E. coli K12 and the dnaB-like function of P1 prophage. J. Mol. Biol. 94, 327-340.

Reuben, C. R. and Gefter, M. L. (1973). A DNA-binding protein induced by bacteriophage T7. Proc. Natl. Acad. Sci. USA 70, 1846-1850.

Reuben, C. R. and Gefter, M. L. (1974). A deoxyribonucleic acid-binding protein induced by bacteriophage T7. Purification and properties of the protein. J. Biol. Chem. 249, 3843-3850.

Richardson, C. C., Romano, L. J., Kolodner, R., LeClerc, J. E., Tamanoi, F., Engler, M. J., Dean, F. B. and Richardson, D. S. (1978). Replication of bacteriophage T7 DNA by purified proteins. Cold Spring Harbor Symp. Quant. Biol. 43, 427-440.

Sancar, A. and Rupp, W. D. (1979) Cloning of uvrA, lexC and ssb genes of Escherichia coli. Biochem. Biophys. Res. Commun. 90, 123-129.

Sevastopoulous, C. G., Wehr, C. T. and Glaser, D. A. (1977). Large-scale automated isolation of Escherichia coli mutants with thermosensitive DNA replication. Proc. Natl. Acad. Sci. USA 74,

3485-3489.

Shapiro, A. L., Vinuela, E. and Maizel, J. V. (1967) Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. 28, 815-820.

Scherzinger, E., Litfin, F. and Jost, E. (1973). Stimulation of T7 DNA polymerase by a new phage-coded protein. Mol. Gen. Genet. 123, 247-262.

Scherzinger, E. and Klotz, G. (1975). Studies of bacteriophage T7 DNA synthesis in vitro. II. Reconstitution of the T7 replication system using purified proteins. Mol. Gen. Genet. 141, 233-249.

Studier, F. W. (1969). The genetics and physiology of bacteriophage T7. Virology 39, 562-574.

Studier, F. W. (1973). Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. 79, 237-248.

Tomizawa, J., Anraku, N. and Iwama, Y. (1966). Molecular mechanism of genetic recombination of bacteriophage. VI. A mutant defective in the joining of DNA molecules. J. Mol. Biol. 21, 247-253.

Tsujimoto, Y. and Ogawa, H. (1977). Intermediates in genetic recombination of bacteriophage T7 DNA. J. Mol. Biol. 109, 423-436.

Tsujimoto, Y. and Ogawa, H. (1978). Intermediates in genetic recombination of bacteriophage T7 DNA. Biological activity and the roles of gene 3 and gene 5. J. Mol. Biol. 125, 255-273.

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 efficiency 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

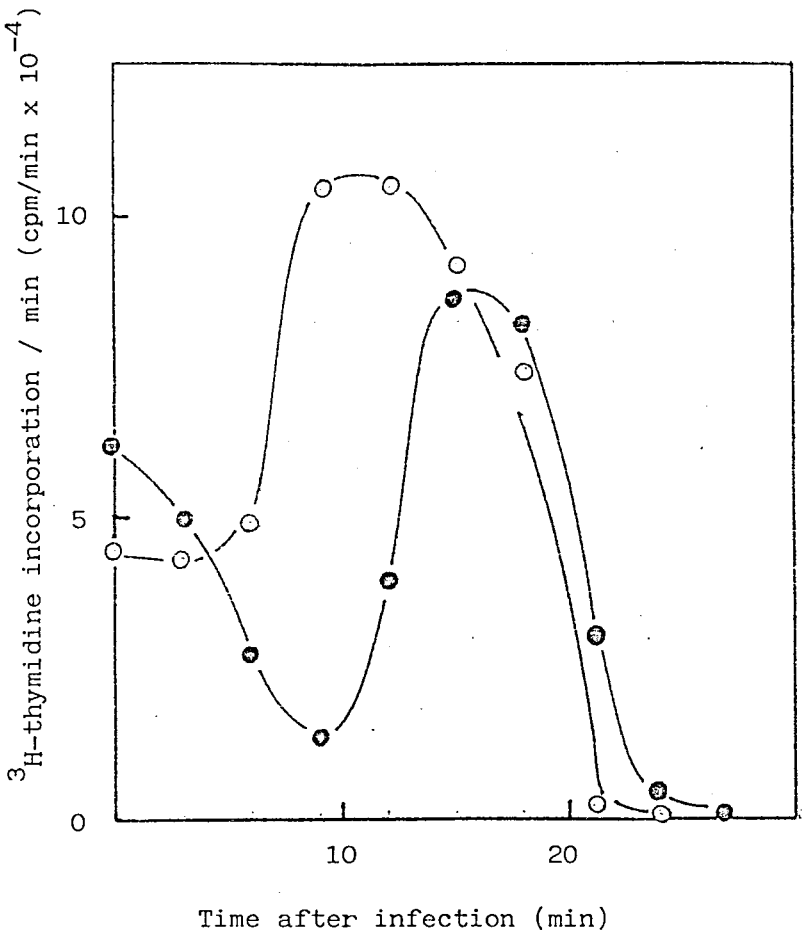

Figure III-8. Time course of the rate of the DNA synthesis after the infection of T7 phage.

Bacteria, CAJ64, were infected with T7 wild-type(○) 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-2*am*233 X UP-2*am*10	38	29.2
*am*233 X *am*10	101	38.3

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

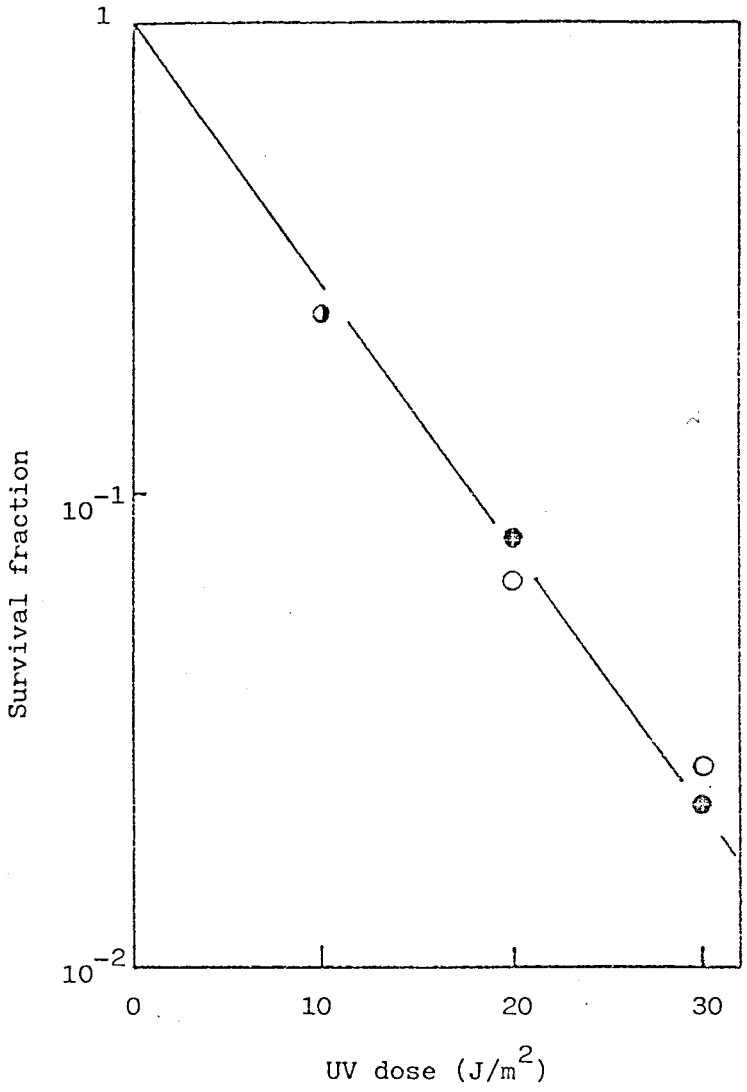

Figure III-9. UV sensitivity of T7 UP-2 phage on CAJ64 bacteria.

T7 wild-type (O) and UP-2 mutant (●) were irradiated with UV light and plated with CAJ64 bacteria.

REFERENCES

Mosig, G., Luder, A., Garcia, G., Dannenberg, R. and Bock, S. (1978). In vivo interactions of genes and proteins in DNA replication and recombination in phage T4. Cold Spring Harbor Sym. Quant. Biol. 43, 501-515.

Reuben, C. R. and Gefter, M. L. (1974) A deoxyribonucleic acid-binding protein induced by bacteriophage T7. Purification and properties of the protein. J. Biol. Chem. 249, 3843-3850.

Sambrook, J. F., Fan, D. P. and Brenner, S.(1967) A strong suppressor specific for UGA. Nature 214, 452-453.

Scherzinger, E. and Klotz, G. (1975) Studies on bacteriophage T7 DNA synthesis in vitro. II. Reconstitution of the T7 replication system using purified proteins. Mol. Gen. Genet. 141, 233-249.

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°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°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°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°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 <u>in vitro</u> DNA synthesis by T7 DNA-binding protein

Stimulation of DNA polymerase [illegible] 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 pyrophospphate 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 <u>et al</u>. (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 polyacrylamide 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 polyacrylamide 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).

RESULTS

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-

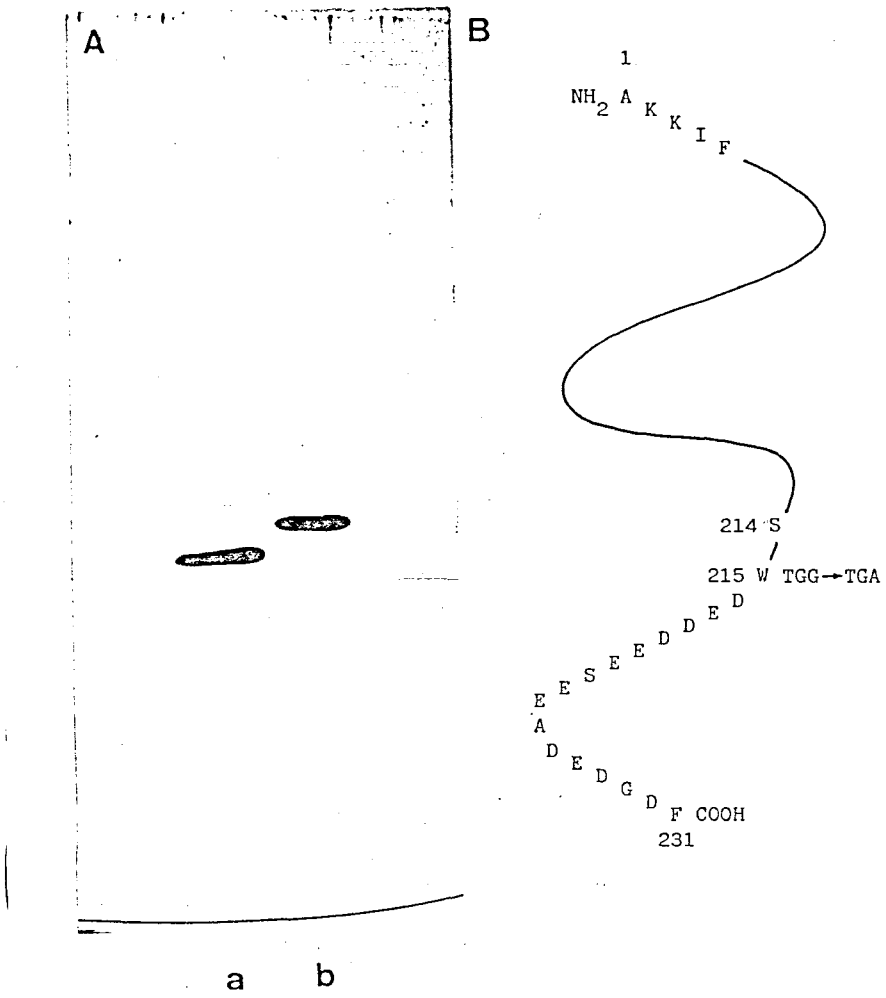

Figure IV-1.

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.

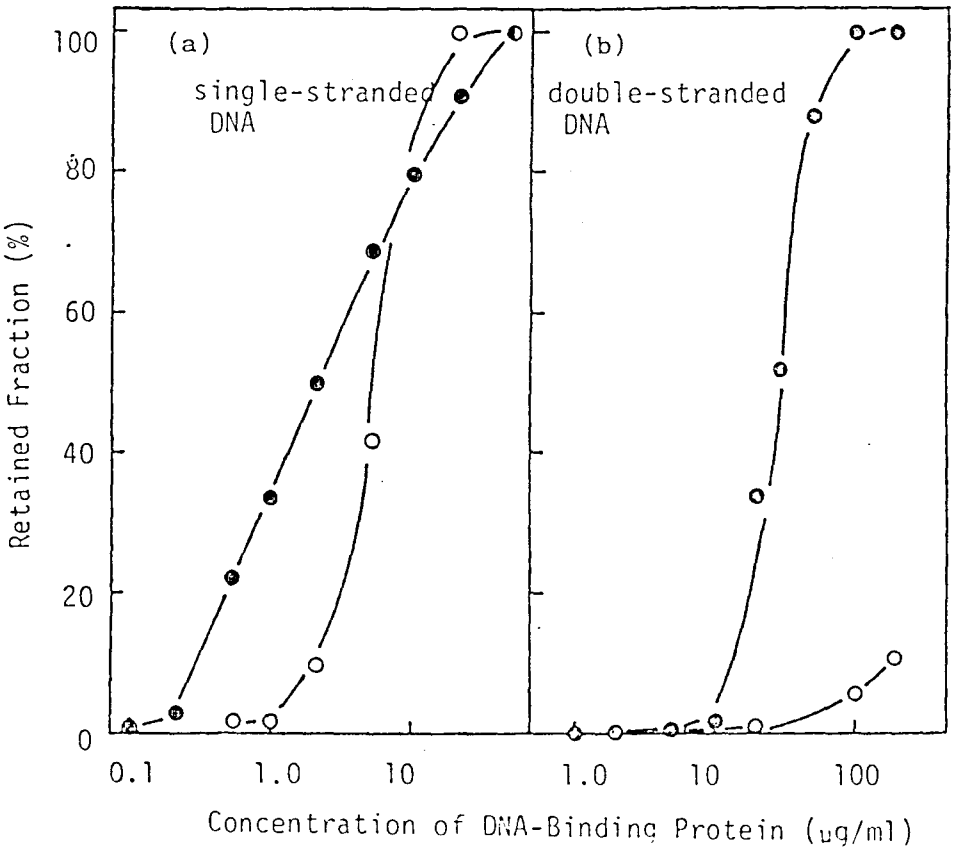

Figure IV-2. DNA binding of mutant protein (●) and wild-type protein (○). ^{3}H-labelled φx174 phage DNA (1 μg/ml) (a) or φx174 RFI DNA (1 μg/ml) (b) was incubated at 30°C with mutant or wild-type protein at indicated concentration for 10 min. After the sample was passed through nitrocellulose filter, the radioactivity retained on the filter was measured.

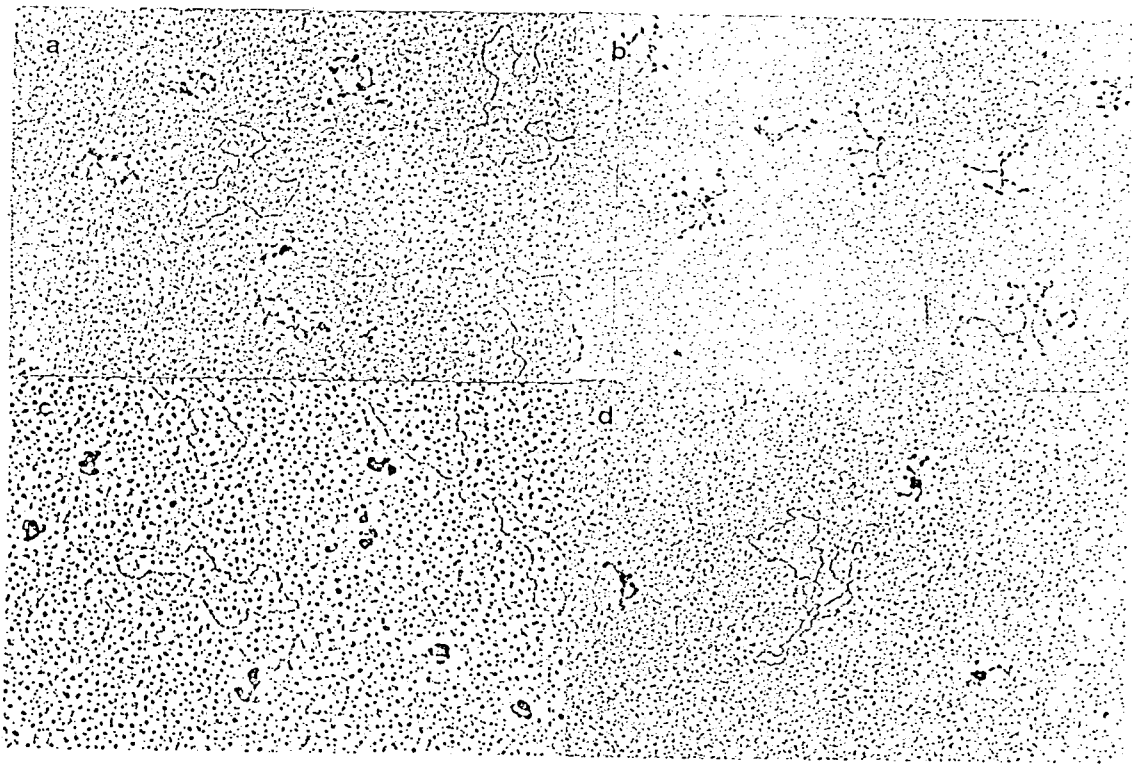

Figure IV-3. Electron microscopy of complexes of ϕx174 DNA with a mutant and a wild-type protein. ϕx174 DNA (2 μg/ml) was incubated with mutant (100 μg/ml) (b, d) or wild-type protein (100 μg/ml) (a, c) at 30°C in the presence (c, d) or absence (a, b) of Mg^{2+}. The samples were fixed with 0.01 M glutaraldehyde 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

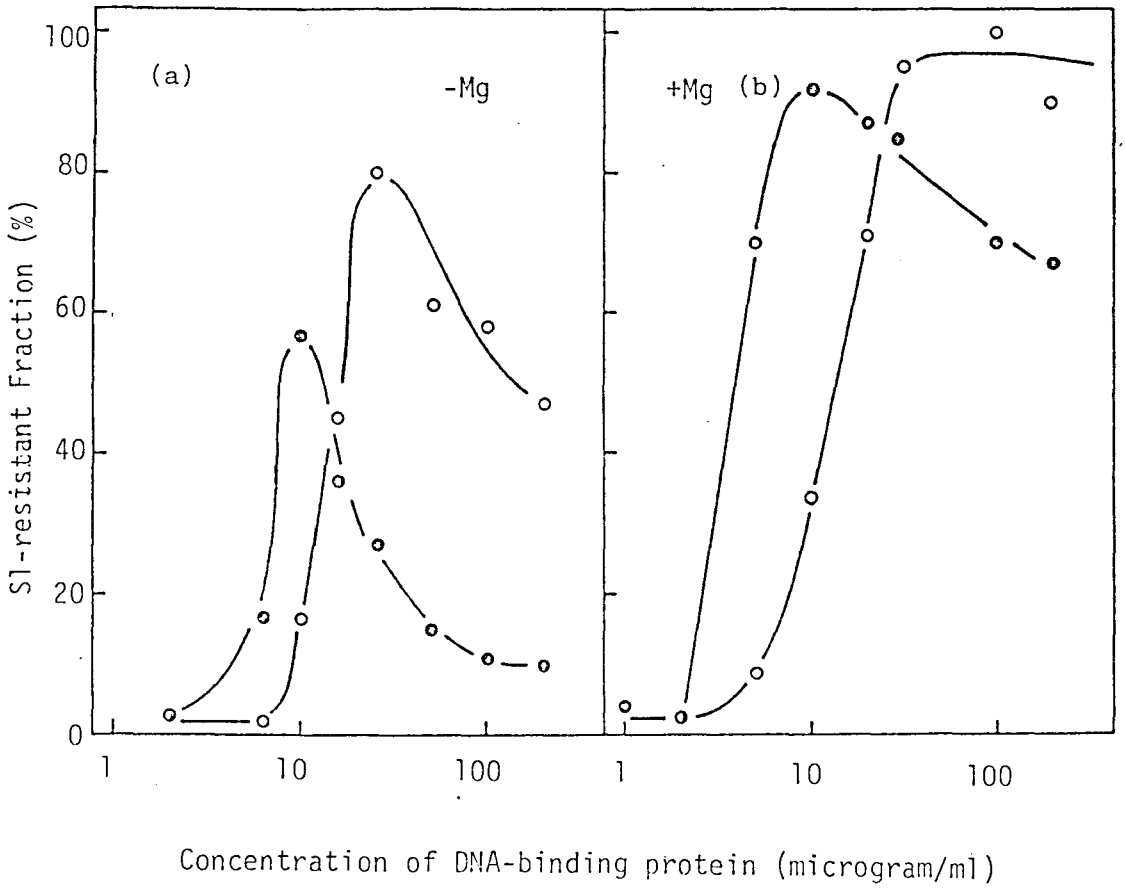

Figure IV-4. Renaturation activity of mutant (●) and wild-type (○) protein in the absence (a) and the presence (b) of Mg^{2+}.

Heat-denatured ^{3}H-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.

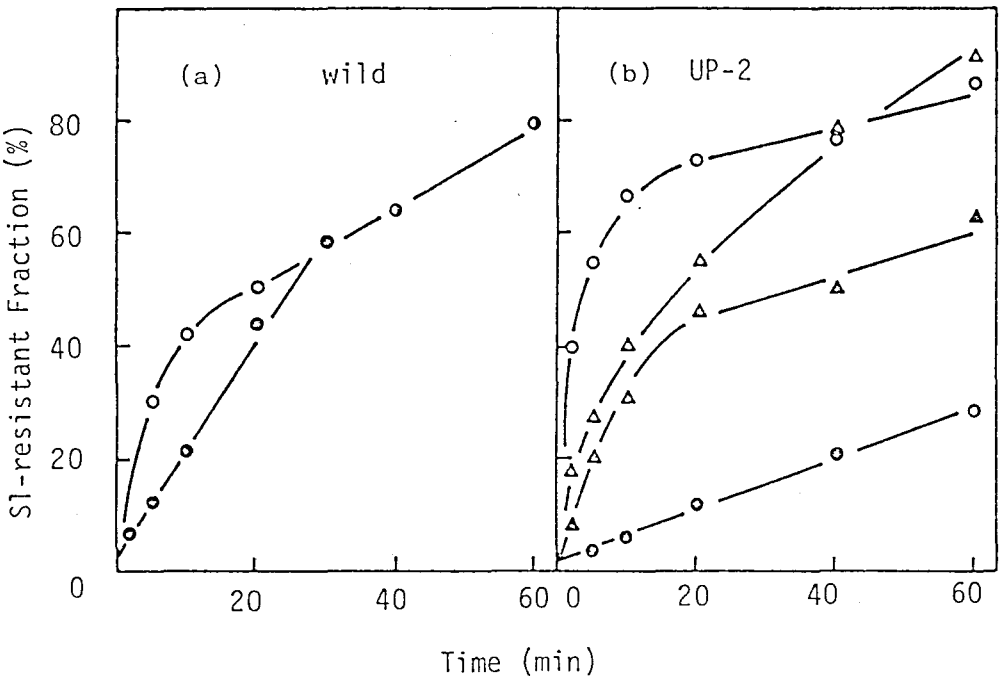

Figure IV-5. Time course of renaturation.

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 (○) 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 (○●) of mutant protein for indicated times in the presence (△○) 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

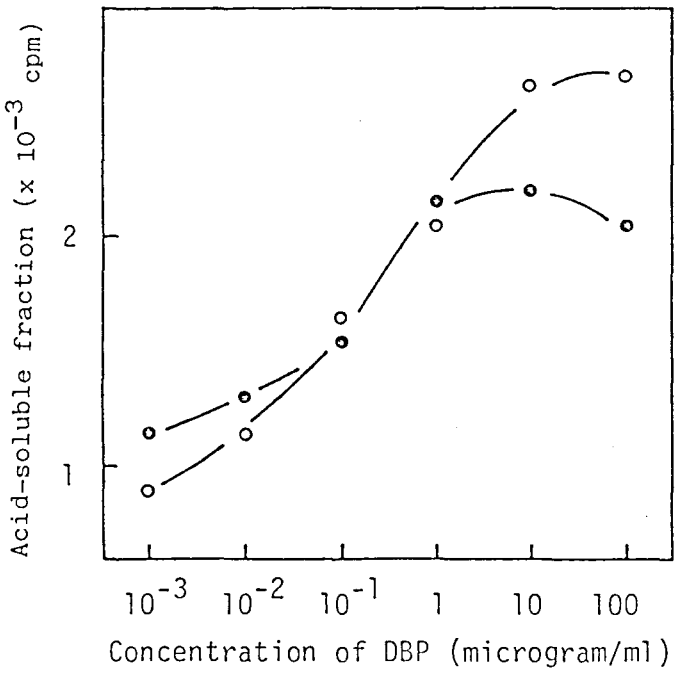

Figure IV-6. Stimulation of T7 exonuclease activity by mutant (●) and wild-type (○) protein. After ^{3}H-labelled T7 DNA (0.5 μg, 9,600 cpm) was incubated at 30°C with 0.1 unit of T7 exonuclease and mutant or wild-type protein at indicated concentration for 30 min, the acid-soluble fraction was measured.

(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).

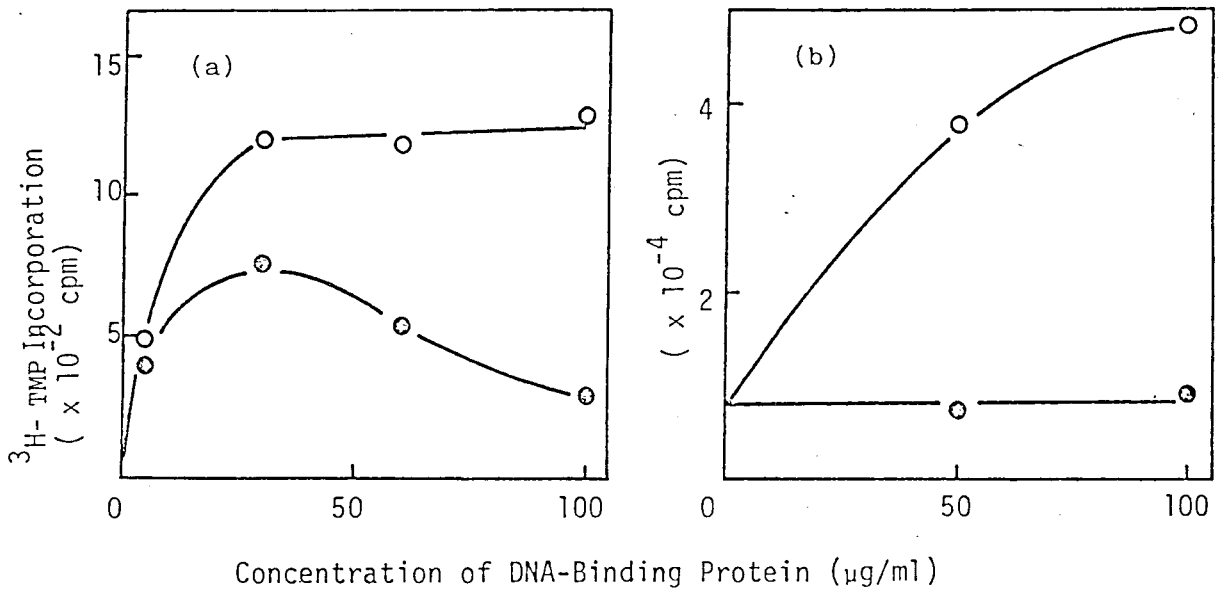

Figure IV-7. Stimulation of DNA synthesis by mutant (●) and wild-type (○) DNA-binding protein.

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.

REFERENCES

Araki, H. and Ogawa, H. (1981a) The participation of T7 DNA-binding protein in T7 genetic recombination. Virology 111, 509-515.

Araki, H. and Ogawa, H. (1981b) A T7 amber mutant defective in DNA-binding protein. Mol. Gen. Genet. 183, 66-73.

Araki, H. and Ogawa, H. (1982) Novel amber mutants of bacteriophage T7, growth of which depends on E. coli DNA-binding protein. Virology in press.

Burke, R. L., Alberts, B. M. and Hosoda, J. (1980) Proteolytic removal of the COOH terminus of the T4 gene 32 helix-destabilizing protein alters the T4 in vitro replication complex. J. Biol. Chem. 255, 11484-11494.

Champoux, J. J. (1978) Proteins that affect DNA conformation . Annu. Rev. Biochem. 47, 449-480.

Davis, R. W., Simon, M. and Davidson, N. (1971) Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. In Methods in Enzymology (Grossman, L. and Moldave, K., eds), vol. 21, pp. 413-428, Academic Press, New York.

Dunn, J. J. and Studier, F. W. (1981) Nucleotide sequence from the genetic left end of bacteriophage T7 DNA to the beginning of gene 4 . J. Mol. Biol. 148, 303-330.

Grippo, P. and Richardson, C. C. (1971) Deoxyribonucleic acid polymerase of bacteriophage T7. J. Biol. Chem. 246, 6867-6873.

Hase, T., Wakabayashi, S., Matsubara, H., Kerstner, L., Oesterhelt, D., Rao, K. K. and Hall, D. O. (1978) Complete amino acid sequence of Halobacterium halobium ferredoxin containing N^{ε} - acetyllysine residue. J. Biochem. 83, 1657-1670.

Ikeda, H. and Tomizawa, J. (1965) Transducing fragments in generalized transduction by phage P1. I. Molecular origin of the fragments. J. Mol. Biol. 14, 85-110.

Jensen, D., Kelly, R. and von Hippel, P. (1976) DNA "melting" proteins. II. Effect of bacteriophage T4 gene 32-protein binding on the conformation and stability of nucleic acid structure. J. Biol. Chem. 251, 7215-7228.

Kolodner, R., Masamune, Y., LeClerc, J. E. and Richardson, C. C. (1978) Gene 4 protein of bacteriophage T7. J. Biol. Chem. 253, 566-573.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Maxam, A. M. and Gilbert, W. (1979) Sequencing end-labeled DNA with base-specific chemical cleavages. In Methods in Enzymology (Grossman, L. and Moldave, K., eds), vol. 65, pp. 499-559, Academic Press, New York.

Moise, H. and Hosoda, J. (1976) T4 gene 32 protein model for control of activity at replication fork. Nature 259, 455-458.

Narita, K. (1970) Hydrazinolysis method of Akabori. In Protein Sequence Determination (Needleman, S. B., ed), pp. 60-69, Springer-Verlag, Berlin.

Newport, J. W., Kowalcyzkowski, S. C., Lonberg, N., Paul, L. S. and von Hippel, P. H. (1980) Molecular aspects of the interactions of T4-coded gene 32-protein and DNA-polymerase (gene 43-protein) with nucleic acids. In Mechanistic Studies of DNA Replication and

Genetic Recombination: ICN-UCLA Symposia on Molecular and Cellular Biology (Alberts, B. M., ed), vol. 19, pp. 483-503, Academic Press, New York.

O'Farrell, P. H. (1975) High resolution two-dimentional electrophoresis of proteins. J. Biol. Chem. 250, 4007-4021.

Ogawa, H., Araki, H. and Tsujimoto, Y. (1978) Recombination intermediates in the extract from T7-infected cells. Cold Spring Harbor Symp. Quant. Biol. 43, 1033-1041.

Pagano, J. S. and Hutchinson III, C. A. (1971) Small, circular, viral DNA: Preparation and analysis of SV40 and ϕx174 DNA. In Methods in Virology (Maramorosch, K. and Koprowski, H., eds), vol. 5, pp. 79-123.

Reuben, C. R. and Gefter, M. L. (1973) A DNA-binding protein induced by bacteriophage T7. Proc. Natl. Acad. SCi. USA 70, 1846-1850.

Reuben, C. R. and Gefter, M. L. (1974) A deoxyribonucleic acid-binding protein induced by bacteriophage T7. J. Biol. Chem. 249, 3843-3850.

Richardson, C. C., Romano, L. J., Kolodner, R., LeClerc, J. E., Tamanoi,F., Engler, M. J., Dean, F. B. and Richardson, D. S. (1978) Replication of bacteriophage T7 DNA by purified proteins. Cold Spring Harbor Symp. Quant. Biol. 43, 427-440.

Sadowski, P. D., Bradley, W., Lee, D. and Roberts, L. (1980) Genetic recombination of bacteriophage T7 DNA *in vitro*. In Mechanistic Studies of DNA Replication and Genetic Recombination: ICN-UCLA Symposia on Molecular and Cellular Biology (Alberts, B. M., ed), vol. 19, pp. 941-952, Academic Press, New York.

Sancar, A., Williams, K. R., Chase, J. W. and Rupp, W. D. (1981) Sequences of the *ssb* gene and protein. Proc. Natl. Acad. Sci. USA 78, 4274-4278.

Sherzinger, E., Litfin, F. and Jost, E. (1973). Stimulation of T7

DNA polymerase by a new phage-coded protein. Mol. Gen. Genet. 123, 247-262.

Sherzinger, E. and Klotz, G. (1975) Studies of bacteriophage T7 DNA synthesis in vitro. II. Reconstitution of the T7 replication system using purified proteins. Mol. Gen. Genet. 141, 233-249.

Shimizu, K., Araki, H. and Ogawa, H. (1982) Genetic recombination between DNA-membrane complexes of bacteriophage T7. manuscript in preparation.

Studier, F. W. (1973) Analysis of bacteriophage T7 early RNAs and proteins on slab gels. J. Mol. Biol. 79, 237-248.

Tsujimoto, Y. and Ogawa, H. (1977) Intermediates in genetic recombination of bacteriophage T7 DNA. J. Mol. Biol. 109, 423-436.

Williams, K. R., LoPresti, M. B., Setoguchi, M. and Konigsberg, W. H. (1980) Amino acid sequence of the T4 DNA helix-destabilizing protein. Proc. Natl. Acad. Sci. USA 77, 4614-4617.

Williams, K. R., Guggenheimer, R., Chase, J. and Konigsberg, W. H. (1981) Physicochemical properties of a limited proteolysis product of the E. coli single-stranded DNA binding protein (SSB). Fed. Proc. Fed. Am. Soc. Exp. Biol. 40, 1731.

Yamagishi, M., Fujisawa, H. and Minagawa, T. (1981) Bacteriophage T3 DNA binding protein interaction with DNA. Virology 109, 148-156.

Yeats, S. M., Schwarz, T. F. R., Ryan, T. P., Carner, I., Hayward, R. S. and McConnell, D. J. (1981) A possible role in transcription for the single-stranded DNA binding protein of bacteriophage T7. Mol. Gen. Genet. 183, 187-191.

Zimmerman, C. L., Appella, E. and Pisano, J. J. (1977) Rapid analysis of amino acid phenylthiohydantoins by high-performance liquid chromatography. Anal. Biochem. 77, 569-573.

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