AN ABSTRACT OF THE THESIS OF

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Title: Organization of T4 Bacteriophage Genes and Gene Products Involved in DNA Precursor Biosynthesis.

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Bacteriophage T4 gene 42 encodes dCMP hydroxymethylase, an enzyme unique to the deoxyribonucleotide metabolism of T-even bacteriophages. To study biochemical and biophysical properties of the enzyme, as well as the interaction of dCMP hydroxymethylase with other DNA precursor biosynthetic enzymes in vitro, availability of large amounts of the enzyme is necessary. Therefore, cloning and overexpression of T4 gene 42 are needed for these studies.

An 1.8-kb Eco RI restriction fragment of a T4 multiple mutant BK536, containing an amber mutation in gene 42, has been cloned into pUC19 plasmid vector in *Escherichia. coli.* Genetic and biochemical examination of the cloned T4 DNA fragment has revealed that it contains the entire gene 42 coding sequence. The cloned amber mutant gene was converted to a wild type gene by site-directed mutagenesis, and the wild type gene 42 was overexpressed with the pT7 expression vector system.

dCMP hydroxymethylase expressed from the cloned gene 42 was purified to homogeneity. The specific activity and N-terminal amino acid sequence of the purified enzyme were determined. The specific

activity of the purified cloned gene product closely agreed with the value of the enzyme purified from phage-infected *E. coli* cells. N-terminal amino acid determination has confirmed the open reading frame of gene 42, deduced from nucleotide sequence of gene 42. Purified dCMP hydroxymethylase has also been used to study its crystal structure and catalytic mechanism by collaboration with other research groups.

In addition, purified dCMP hydroxymethylase was immobilized on Affi-Gel to prepare an affinity column, which was used to study protein-protein interaction among dNTP biosynthetic enzymes and with other DNA metabolic proteins. The proteins which specifically interact with dCMP hydroxymethylase were identified by SDS polyacrylamide gel electrophoresis followed by fluorography, Western blotting, and two dimensional gel electrophoresis.

It was earlier found that T4-encoded thymidylate synthase and dihydrofolate reductase function not only in deoxyribonucleotide biosynthesis but are also structural components of the phage baseplate. Two deletion mutants containing deletion in the *td* gene, encoding thymidylate synthase, and the *frd* gene, encoding dihydrofolate reductase, were carefully characterized, and used to re-evaluate structural role of these two enzymes.

ORGANIZATION OF T4 BACTERIOPHAGE GENES AND GENE PRODUCTS INVOLVED IN DNA PRECURSOR BIOSYNTHESIS

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ORGANIZATION OF T4 BACTERIOPHAGE GENES AND GENE PRODUCTS INVOLVED IN DNA PRECURSOR BIOSYNTHESIS

I General Introduction

Bacteriophage T4 has played a very important role in the history of biochemistry as well as molecular biology. Studies with T4 phage as a model system have yielded invaluable information for exploring enzymology and regulation of DNA metabolism. Infection of *Escherichia coli* by bacteriophage T4 induces synthesis of an ensemble of virus-coded DNA precursor biosynthetic enzymes. These enzymes, together with a small number of host enzymes, generate a specialized deoxyribonucleotide biosynthetic pathway in phage-infected cells. It has been proposed that DNA precursor biosynthetic enzymes associate to form a multienzyme complex which can efficiently provide deoxynucleoside triphosphates to the DNA replication apparatus and balance the concentration of each dNTP at replication sites (reviewed by Mathews et al., 1979). In addition, some of the dNTP biosynthetic enzymes have been found also to play structural roles in the T4 phage life cycle.

By employing the bacteriophage T4 system, the goal of the research presented here is to develop a system for further studying interactions among DNA precursor biosynthetic enzymes, and the correlation between DNA precursor biosynthesis and DNA replication. The functions of phage-encoded thymidylate synthase and dihydrofolate reductase as structural elements of the virus particle will

also be further investigated.

1. Physical Properties of Bacteriophage T4

Bacteriophage T4 is an *Escherichia coli* virus. The virion consists of a large head and a complex tail. The head is filled with double-stranded DNA, and the tail consists of a tail tube surrounded by sheath protein, a baseplate, and fibers extending from the baseplate. T4 tail baseplate is composed of at least 16 different proteins with a complicated, symmetrical, hexapartite construction. The baseplate changes its configuration during the irreversible attachment process. One special feature of T4 phage infection is that the contraction of its sheath protein can mediate the injection of viral DNA into the *E. coli* cell (Goldberg, 1983).

The double-stranded T4 DNA has genomic a size of 166 kilobase pairs, which has been mapped and largely sequenced. The phage DNA codes for over 200 gene products; one half of which are involved in deoxyribonucleotide biosynthesis, DNA replication and the regulation of the gene expression during infection. The remaining genes code for structural components and the enzymes catalyzing phage assembly (Mosig, 1983).

2. T4 Life Cycle

The T4 phage life cycle is initiated by adsorption of phage to the

host cell wall via the long tail fibers. The specialized structure of the T4 virion permits it to recognize and infect its host efficiently. The plating efficiency of T4 is much higher than those of other bacteriophages such as λ, T5 or T7. The highly efficient infection of T4 phage is the result of its complicated tail, whose sheath contracts about a central tube which conducts DNA from the phage head into the host cell cytoplasm (Goldberg, 1983).

Once T4 DNA gets inside of an *E. coli* cell, a sequential gene expression program begins (Rabussay and Geiduschek, 1977; Brody et al., 1983; Christernsen and Young, 1983). A class of genes, called immediate early genes, is transcribed by unmodified *E. coli* RNA polymerase, starting a few seconds after T4 infection. The second class of genes, called delayed early genes, is expressed two to three minutes after infection, by ADP-ribosyl-modified *E. coli* RNA polymerase. Most immediate and delayed early genes can be expressed through both early mode promoters and middle mode promoters, and those genes encode the regulatory proteins of phage transcription and translation, and the enzymes involved in deoxyribonucleotide biosynthesis and DNA replication. T4 early genes also encode two endonucleases for degrading *E. coli* chromosomal DNA and supplying 4 building blocks for T4 DNA biosynthesis. Synthesis of these early enzymes ceases at about 12 minutes after infection at 37 C.

Beginning at about 5 minutes after phage infection, T4 DNA replication begins, along with the transcription of another class of genes, which code for the so-called late proteins. The late proteins

include the virion structural proteins and the enzymes for phage assembly, packaging of DNA into phage heads, and lysis of the host cell. T4 late gene promoters have a consensus sequence different from the *E. coli* promoter sequence. Presumably to allow recognition of these promoters, modification of the host RNA polymerase by phage-encoded proteins, gene product(gp)33, gp45 and gp55, is required for the expression of the late genes. In addition, the onset of phage DNA replication is necessary for late gene expression (Rabussay, 1983; Geiduschek et al., 1983).

The phage heads are made in one pathway, and the baseplates of the tail are made in two others which then converge into a common pathway for tail assembly. The heads are then filled with DNA and joined to the tails, and to the tail fibers, which are synthesized in another pathway (Black and Showe, 1983; Berget and King, 1983; Wood and Crowther, 1983). The process occurs so rapidly that an infected cell can make at least 200 particles by 20 to 30 minutes after infection at 37 C in rich medium.

3. Special Features of T4 Deoxyribonucleotide Metabolism

Bacteriophage T4 induces most of the deoxyribonucleotide biosynthetic enzymes needed to supply dNTPs for phage DNA replication during infection (review by Mathews and Allen, 1983). To meet the high rate of DNA synthesis in phage-infected cells, T4 phage not only duplicates some of the host functions of deoxyribonucleotide

biosynthesis, but it also induces endonucleases specifically for degradation of host genomic DNA and supplies the major source of the dNTPs for phage DNA replication. In addition, phage infection induces several enzymes which are unique to the infected cell. The phage-encoded enzymes, together with two host enzymes, namely nucleoside diphosphate kinase and dAMP kinase, create new deoxyribonucleotide metabolic pathway during phage infection, as shown in Figure I-1. Phage-coded enzymes, the reactions catalyzed by the enzymes, and some of their physical properties are listed in Table I-1. Some of these enzymes duplicate host functions in the pathway, including ribonucleotide reductase, thymidylate synthase and dihydrofolate reductase. The phage-induced enzymes have different physical properties and allosteric regulation from the corresponding host enzymes. Enzymes unique to the phage infection include dCMP hydroxymethylase and dNMP kinase. In addition to supplying high concentrations of deoxyribonucleotides in T4-infected cell, phage-encoded enzymes also manipulate the composition and the size of each deoxynucleotide pool to allow adaptation to the nucleotide composition of the phage DNA (66% AT). Those enzymes will be discussed in detail below with respect to their specific functions in T4 infection.

4. Deoxyribonucleotide Biosynthesis Complex

DNA replication is more closely coordinated with the synthesis of

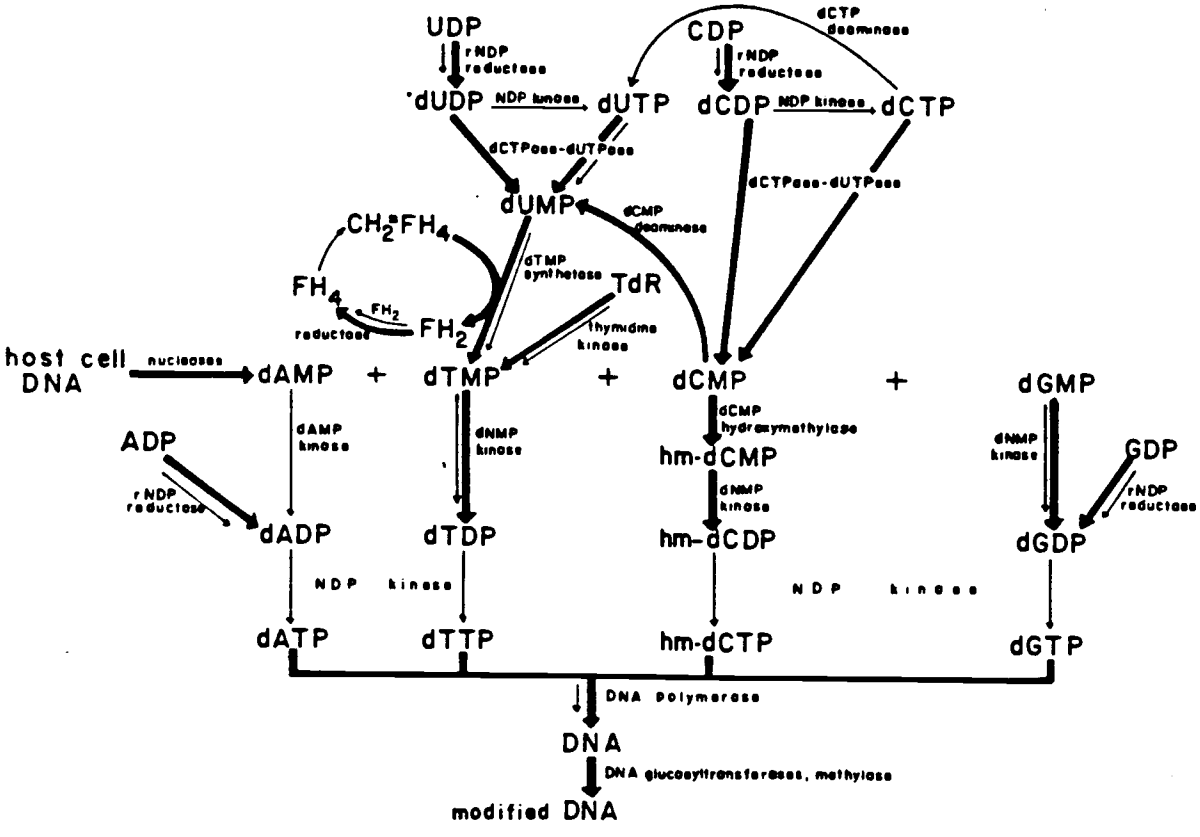
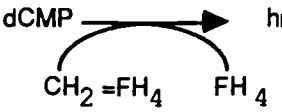
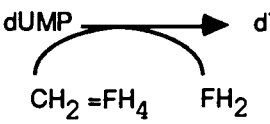
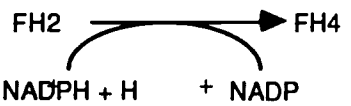
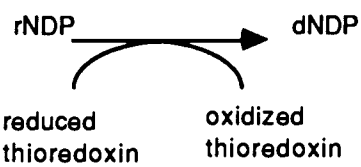
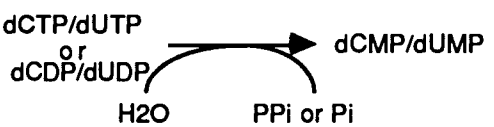
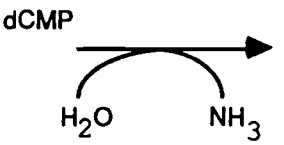


Figure I-1. Reactions of DNA precursor biosynthesis in T4 phage-infected *E. coli*. Reactions catalyzed by phage-induced and preexisting host cell enzymes are represented by heavy and light arrows, respectively (Mathews and Allen, 1983).

Table I-1. T4-encoded enzymes of deoxyribonucleotide metabolism.

Enzyme	Reaction	Mol. Wt (subunit)	Gene
dCMP hydroxymethylase		28.3 kD	42
Thymidylate synthase		33 kD	td
Dihydrofolate reductase		23 kD	frd
Ribonucleotide reductase		85/35 kD	nrdA/nrdB
dNMP kinase		22 kD	1
dCTPase/dUTPase		15 kD	56
dCMP deaminase		20 kD	cd

its precursors, deoxyribonucleotides, than are other macromolecular biosynthetic process. Two aspects of procaryotic DNA replication are in accord with this statement(Mathews and Sinha, 1982): first, DNA replication uses specialized precursors, deoxynucleoside triphosphates, which are rarely used for other metabolic processes; Second, DNA replication is a very rapid process, 850 nucleotides incorporated per second at 37 C, even though the affinity of the replication apparatus for deoxynucleotides is low. For close coordination of deoxynucleotide biosynthesis with the DNA replication apparatus, the enzymes involved in dNTP biosynthesis could associate with each other to form a multienzyme complex and efficiently provide dNTPs at replication fork, as proposed some years ago. Figure I-2 shows a model of a deoxynucleotide biosynthesis complex coupled to a DNA replication fork in a procaryotic system. In addition to maintaining high local concentrations of dNTPs at a replication site, the multienzyme complex can also balance the synthesis of each of the four dNTPs at rates corresponding to the nucleotide composition of DNA. T4 phage system has played an indispensable role in studies of the deoxynucleotide biosynthesis complex.

In 1970s, the research groups of C. K. Mathews and G. R. Greenberg first proposed that the deoxynucleotide biosynthesis complex is integrated with the replicative machinery, and it exists in the T4-infected *E. coli* cell (reviewed by Mathews et al., 1979). This organization would allow deoxyribonucleotides to be channeled directly at the site of DNA synthesis. Therefore, there could be two distinct dNTP pools in T4-infected cells. A pool with high dNTP

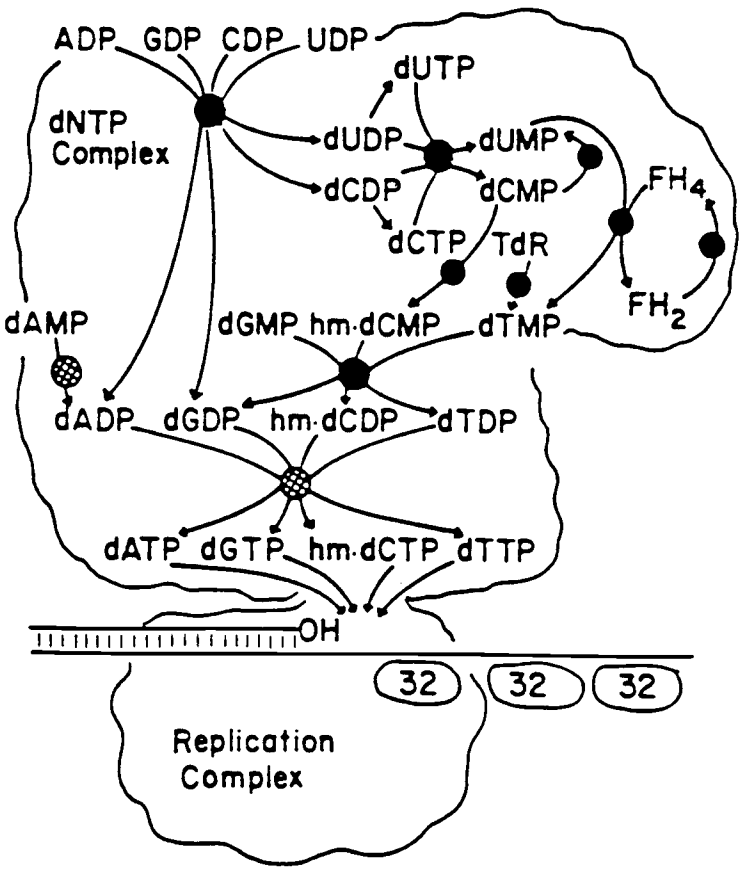


Figure I-2. Speculative view of the T4 deoxynucleotide biosynthesis complex coupled to DNA replication complex. Closed circles denote phage-coded enzymes; cross-hatched circles denote *E. coli* enzymes (Mathews and Allen, 1983).

concentration would be generated and turned over at replication site, and a less highly localized pool could be used for DNA repair and other processes. Various studies (Mathews et al., 1979) indicated that dNTP pools are indeed compartmentalized in both T4-infected and uninfected *E. coli*, although some mixing between these pools does occur.

An aggregate of the deoxyribonucleotide biosynthetic enzymes has been observed in cell extracts of T4-infected *E. coli* cells (Moen et al., 1988), and a multienzyme complex that synthesizes dNTPs from either deoxyribonucleoside monophosphates or ribonucleoside diphosphates has been isolated. This "dNTP synthetase" complex has a molecular weight about 1,500 kD, determined after several hundredfold purification. Eight phage-encoded enzyme activities and two enzymes of bacterial origin were detected in the purified complex.

Several lines of evidence, in addition to co-fractionation of activities, support the conclusion that the aggregate as isolated represents a specific multienzyme complex for DNA precursor biosynthesis. It was observed that the enzymatic activities in the complex are kinetically coupled. Furthermore, phage mutation(s) defective in T4 ribonucleotide reductase led to disruption of the dNTP synthase complex (Moen et al., 1988). More recently, it has been found that amber mutation at the amino terminus of the T4 dCMP hydroxymethylase gene also led to the disruption of the complex (C. Thylen and C. K. Mathews, manuscript in preparation).

5. Modification of T4 Phage DNA

T4 DNA has two unique forms of modification, hydroxymethylation of dCMP, which occurs at the nucleotide level, and glucosylation of the hydroxymethylated DNA, which ocurrs at the DNA level, as observed in other T-even phages. A major role of these DNA structural alterations appears to be a protective one, facilitating the T-even phage lytic life cycle. During T4 infection, phage-induced endonucleases specifically degrade cytosine-containing DNA, such as *E. coli* genomic DNA. Hydroxymethylation of the phage DNA allows T4 to distinguish self (phage) from non-self (*E. coli*) DNA and protects its own DNA from phage-induced endonuclease degradation (Warner and Snustad, 1983). The second level of the modification, glucosylation of the hydroxymethylated DNA, further protects phage DNA from an *E. coli* endonuclease which specifically attacks hydroxymethylated DNA (Revel, 1983).

T4-induced enzymes responsible for the modifications include dCTPase/dUTPase, dCMP hydroxymethylase, dNMP kinase and DNA glucosyltransferases. Figure I-3 shows the enzymatic pathway of the synthesis of the modified DNA. By cleaving dCTP to dCMP, dCTPase actually has two functions: 1, it prevents incorporation of dCTP into T4 DNA by diminishing dCTP concentration; 2, it supplies dCMP for the production of hydroxymethyl dCMP (Price and Warner, 1969). The hydroxymethylation reaction is catalyzed by the T4 gene 42 product, dCMP hydroxymethylase, and the synthesized hydroxymethyl dCMP

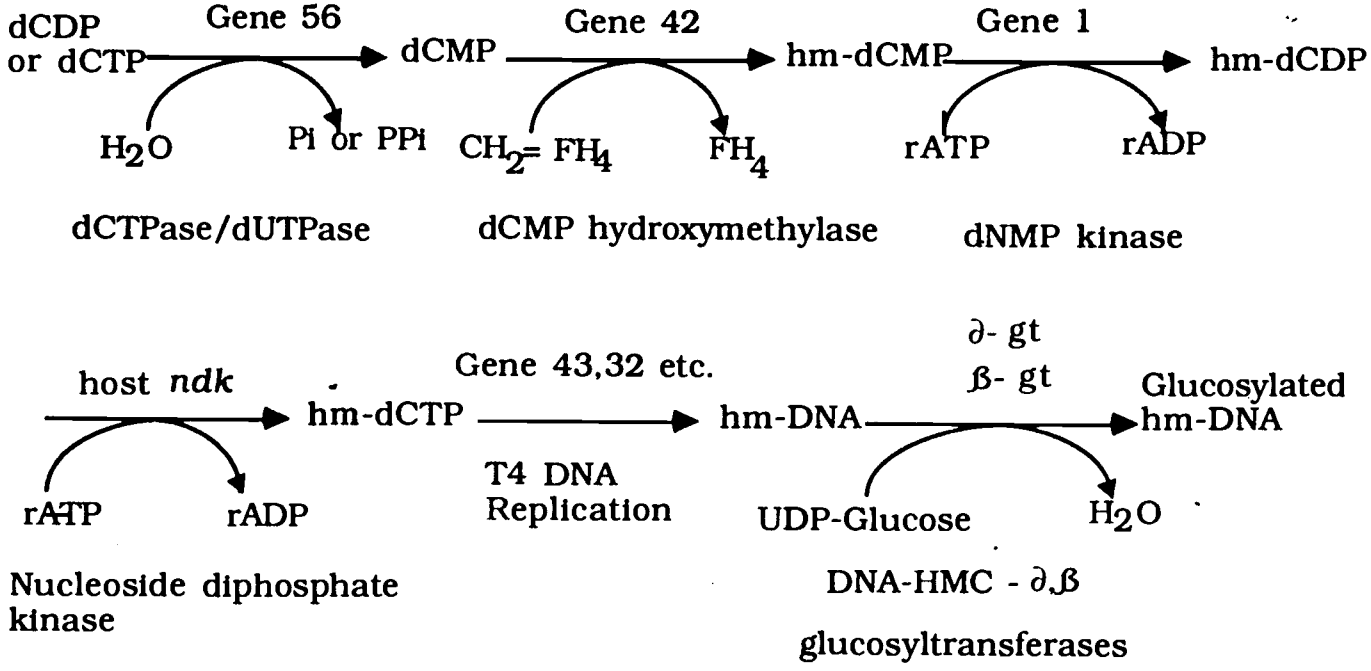


Figure I-3. Enzymatic reactions involved in the synthesis of hydroxymethylated and glucosylated T4 DNA.

(hm-dCMP) is further phosphorylated into the deoxynucleoside triphosphate by T4 dNMP kinase and *E. coli* NDP kinase, and than incorporated into DNA. Since hm-dCMP is not a substrate of host dCMP kinase, T4 dNMP kinase and dCMP hydroxymethylase are essential for hm-dCMP biosynthesis; inactivation of either enzyme prevents phage DNA replication, and is lethal for phage growth. Glucosylation of the hydroxymethylated T4 DNA is catalyzed by T4-induced glucosyltransferase(s).

6. Biochemical Significance of Bacteriophage T4-induced dCMP Hydroxymethylase

In 1957, Flaks and Cohen discovered that infection of *Escherichia coli* with T-even bacteriophages leads to the appearance of a new enzyme activity, the hydroxymethylation of deoxycytidylate. The enzyme, dCMP hydroxymethylase, was the first enzyme discovered to be induced by phage infection. It was also the first demonstration that virus infection can direct the development of novel metabolic pathways in phage-infected cells. Later studies revealed that phage-induced enzymes are synthesized de novo after phage infection (Mathews et al., 1964). In addition, the enzyme was also one of the first essential gene products in T4 to have its structural gene identified and mapped (Wiberg and Buchanan, 1964).

The roles of dCMP hydroxymethylase in DNA precursor biosynthesis and DNA replication, as well as interactions of the enzyme

with other proteins, has been studied extensively over the years. In 1968, Chiu and Greenberg found that gently lysed T4-infected *E. coli* contained a rapidly sedimenting form of dCMP hydroxymethylase. This discovery, along with other studies with dCMP hydroxymethylase and its gene (Collinsworth and Mathews, 1974; North et al., 1976; Wovcha et al., 1973) constituted strong evidence supporting a model of functional compartmentation of DNA precursor biosynthesis in procaryotic organisms. Since then, dCMP hydroxymethylase has been used as a system to further pursue the study of multienzyme complexes and studies of DNA replication fidelity in procaryotic systems (Chiu and Greenberg, 1973; Drake, 1973; Williams and Drake, 1977; Chao et al., 1977; Tomich et al., 1974). More recently, Carson and Overvatn (1986) showed that the activity of T4-induced endonuclease IV, but not that of endonuclease II, was stimulated in the presence of a wild-type dCMP hydroxymethylase, also when no HmCyt was incorporated into phage DNA, suggesting the possibility of direct endonuclease IV-dCMP hydroxymethylase interaction.

7. Enzymatic Properties of dCMP Hydroxymethylase and Its Gene Structure

The reaction catalyzed by the enzyme is as follows;

dCMP + 5,10-methylenetetrahydrofolate ------>

5-hydroxymethyl-dCMP + tetrahydrofolate.

Some years ago, the T4 enzyme was purified to homogeneity from phage-infected *E. coli* cells, and some physical properties and

enzyme kinetic properties were determined (North and Mathews, 1977). The enzyme consists of two identical subunits with a molecular weight of 28 kilodalton each, determined by SDS gel electrophoresis and analytical ultracentrifugation.

Recently, the structural gene for deoxycytidylate hydroxymethylase, gene 42, from three T-even phages has been sequenced (Lamm et al., 1988). The sequencing has revealed a high degree of homology among the T-even phages in this gene. The enzymes of the three phages are composed of an identical number of amino acid residues. A small number of base changes in DNA sequence leads to only six non-homologous amino acid replacements in the enzymes of T2 and T6 relative to the enzyme of phage T4. Most of the changes occur within the carboxyl terminal part of the enzyme, possibly implying that this part of the protein does not play an important role in the enzymatic reaction. Molecular weight of the enzyme, deduced from the nucleotide sequence of the gene 42 open reading frame, agrees with that determined earlier with the purified protein from T4-infected *E. coli* cells.

8. Relationship of dCMP Hydroxymethylase and Thymidylate Synthase

Numerous chemical and enzymatic studies have revealed that enzymes which catalyze electrophilic substitution reactions at the 5 position of the pyrimidine heterocycle proceed by the general

mechanism shown in Figure I-4 (Starzyk et al.,1982; Pogolotti and Santi, 1977). According to this mechanism, a nucleophilic catalyst of the enzyme adds to the 6 position of the heterocycle to produce a negative charge at the 5 position and activate what is otherwise an inert carbon for reaction with an electrophile (R^+). Subsequent abstraction of the proton at carbon-5 of intermediate 3 and - elimination provide the product and the active enzyme. Enzymes proposed to proceed by this mechanism include thymidylate synthase, dUMP hydroxymethylase (encoded by phages whose DNA contains 5-hydroxymethyl uracil), dCMP hydroxymethylase, and possibly the DNA-cytosine methyltransferase and pseudouridine synthetase.

Figure I-5 shows the enzymatic reactions catalyzed by thymidylate synthase and dCMP hydroxymethylase. Both enzymes catalyze their respective reactions by transferring a functional group to the 5 position of the pyrimidine heterocycle, and 5,10-methylene tetrahydrofolate is used in both reactions as the transferred group donor. The differences between the two reactions are that dCMP hydroxymethylase transfers a hydroxymethyl group to dCMP and 5.10-methylene tetrahydrofolate is not oxidized in the hydroxymethylation reaction, while thymidylate synthase transfers a methyl group to the pyrimidine ring of the dUMP and the cofactor is oxidized to dihydrofolate in the synthesis of dTMP.

In addition to the similarity of the reactions catalyzed by the two enzymes, nucleotide sequencing data of the genes coding for the enzymes also reveal a significant homology between thymidylate

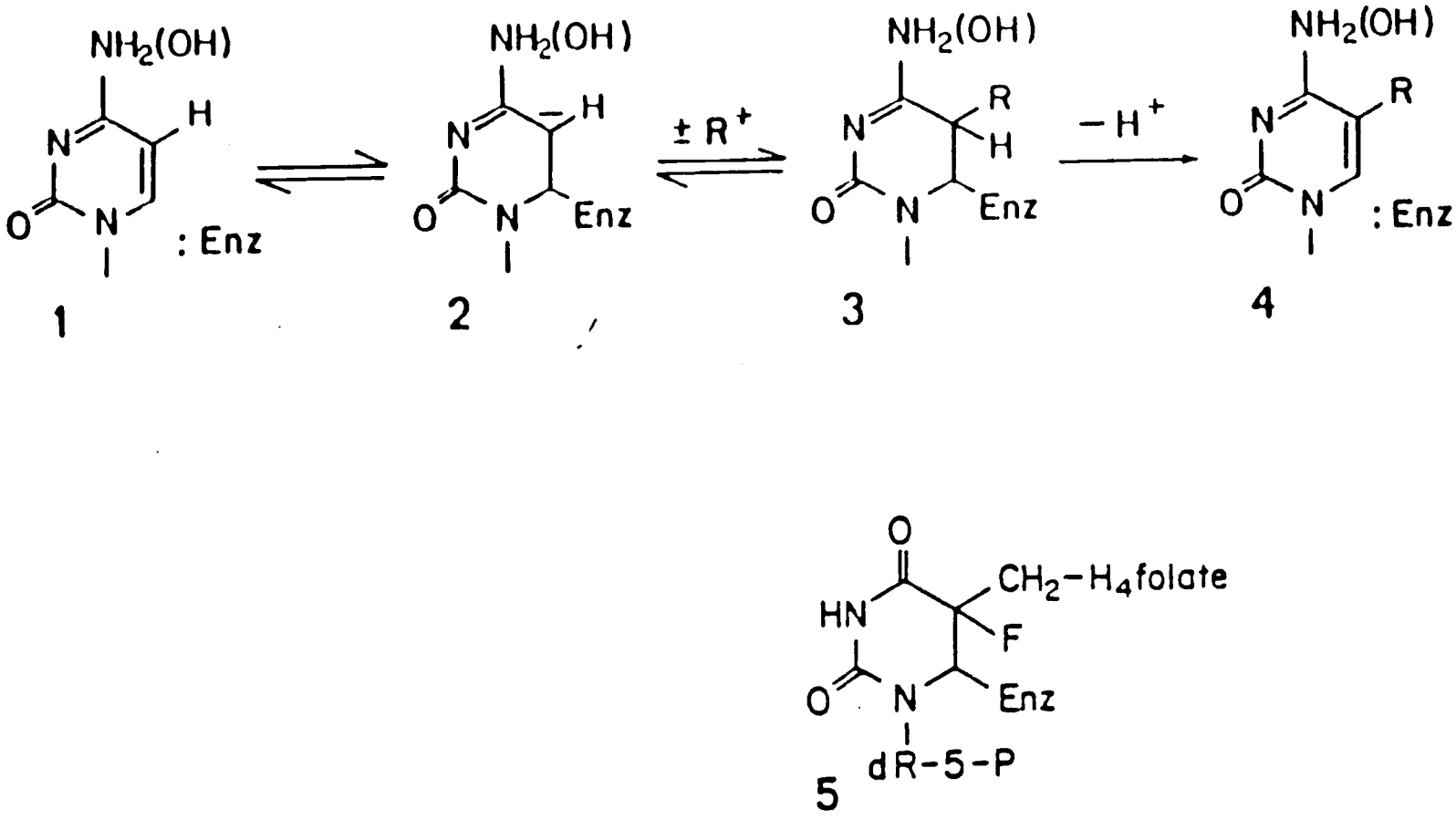


Figure I-4. Mechanism of electrophilic substitution at the 5 position of the pyrimidine heterocycle (Santi, et al., 1983).

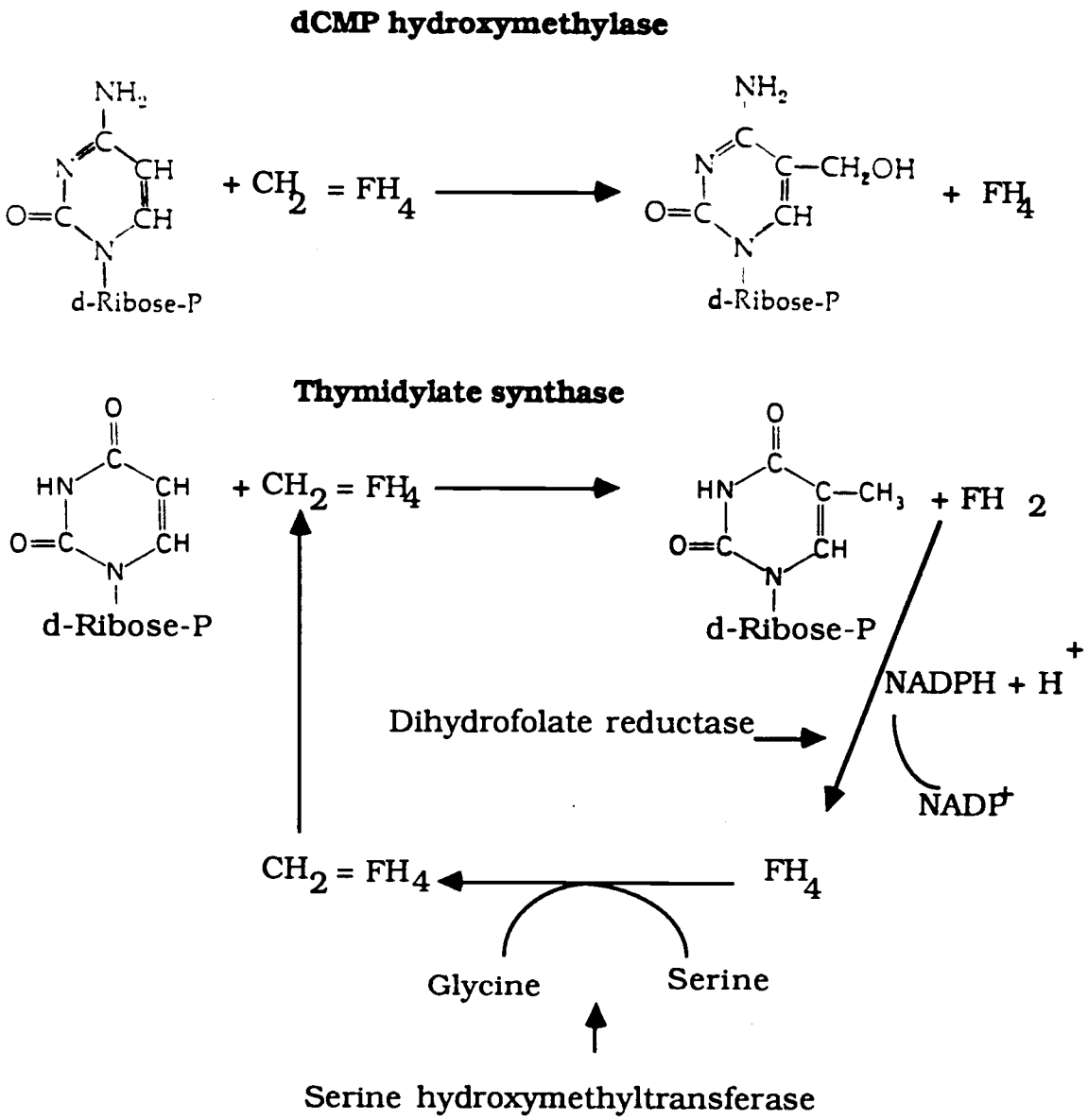


Figure I-5. Enzymatic reactions catalyzed by dCMP hydroxymethylase and thymidylate synthase.

synthase and dCMP hydroxymethylase (Lamm et al., 1988; Belfort et al., 1983). A comparison of the primary structure of hydroxymethylase and thymidylate synthase reveals that a number of seemingly important amino acid positions, including several in the active center of the synthase, are identical (Lamm et al.,1988).

In recent years, 5-methylcytosine residues in DNA have been implicated as having an important role in the control of eucaryotic gene expression (Razin and Riggs, 1980). Consequently, there has been much interest in cytosine analogs such as 5-azacytidine (5-aza-C) and 5-fluorocytosine (5-fluoro-C), which, when incorporated into DNA, inhibit methylation and profoundly affect gene expression and differentiation (Taylor and Jones, 1982). Understanding of the inhibition mechanism of DNA-cytosine methyltransferase by 5-azacytidine and 5-fluorocytosine is very important to studies of eucaryotic gene expression. The inhibition mechanism of the thymidylate synthase by 5-fluorodeoxyuridylate, in the presence of the folate cofactor, 5,10-methylene tetrahydrofolate, is well studied and understood. Through the sequence of conversions described above, a sulfhydryl group of the enzyme adds to the nucleotide analog to form a stable covalent complex having structure 5 shown in Figure I-4. The complex is analogous to the steady state intermediate 3 of the normal enzymatic reaction, except for the presence of a stable carbon-fluorine bond at the 5 position, which prevents its subsequent conversion to products. Since it has been proposed that all enzymes which catalyze electrophilic substitution reactions at the 5 position of the pyrimidine heterocycle proceed by the general mechanism, it is likely that the

other enzymes share a common inhibition mechanism by their respective inhibitors. In other words, the inhibition of DNA-cytosine methyltransferase by 5-aza-C and 5-fluoro-C might proceed by the same mechanism as that of thymidylate synthase by 5-fluoro-dUMP. Studies with DNA-cytosine methyltransferase have proved that the prediction was right (). To further test the hypothesis, dCMP hydroxymethylase should serve as a model system.

9. Significance of Cloning of T4 Gene 42 and Overexpression of dCMP Hydroxymethylase in *E. coli* Cell

For further studies of the biochemical and biophysical properties of dCMP hydroxymethylase, availability of its cloned gene and large amounts of the enzyme are necessary. To study the function of the amino acid residues involved in the catalytic and substrate binding reactions of the enzyme, the most informative strategy is site-directed mutagenesis, by which the native residues are replaced with different ones through mamipulating the sequence at the DNA level. This kind of study requires the availability of the cloned gene and controlled expression of the enzyme.

More important, a large amount of the enzyme is needed to study protein-protein interactions of the deoxynucleotide biosynthesis complex. As mentioned earlier, several approaches have been undertaken to study the multienzyme complex, and one powerful strategy is to do *in vitro* experiments with the purified enzymes.

Such experiments include reconstitution of the multienzyme complex *in vitro* and affinity chromatography. Several laboratories have cloned and expressed T4 genes encoding the deoxynucleotide biosynthetic enzymes, such as the *frd* gene encoding dihydrofolate reductase, the *td* gene encoding thymidylate synthase, gene 56, encoding dUTP/dCTPase, and gene 1, encoding dNMP kinase. Since dCMP hydroxymethylase is proposed as one component of the multienzyme complex, overexpression of the enzyme is needed for these studies.

10. Strategy For Cloning Bacteriophage T4 Genes From Modified T4 DNA

Modification of T4 phage DNA makes cloning its genes very difficult. Hydroxymethylated and glucosylated T4 DNA is resistant to the digestion of commonly used restriction endonucleases. So far only five restriction enzymes are known that can digest the fully modified T4 DNA, but some of them have difficulty in achieving complete digestion. For cloning purposes, a T4 strain BK 536, with multiple mutations in its genome, was generated to produce cytosine-containing DNA instead of modified T4 DNA (Kutter and Snyder, 1983). The cytosine-containing T4 DNA from BK536 can be digested by most commonly used restriction enzymes and is widely used for the cloning of T4 genes. The mutations in BK536 are shown in Figure I-6. The first two mutations in BK 536 are in the denA and denB genes, which code for T4 endonuclease II and endonuclease IV. Inactivation

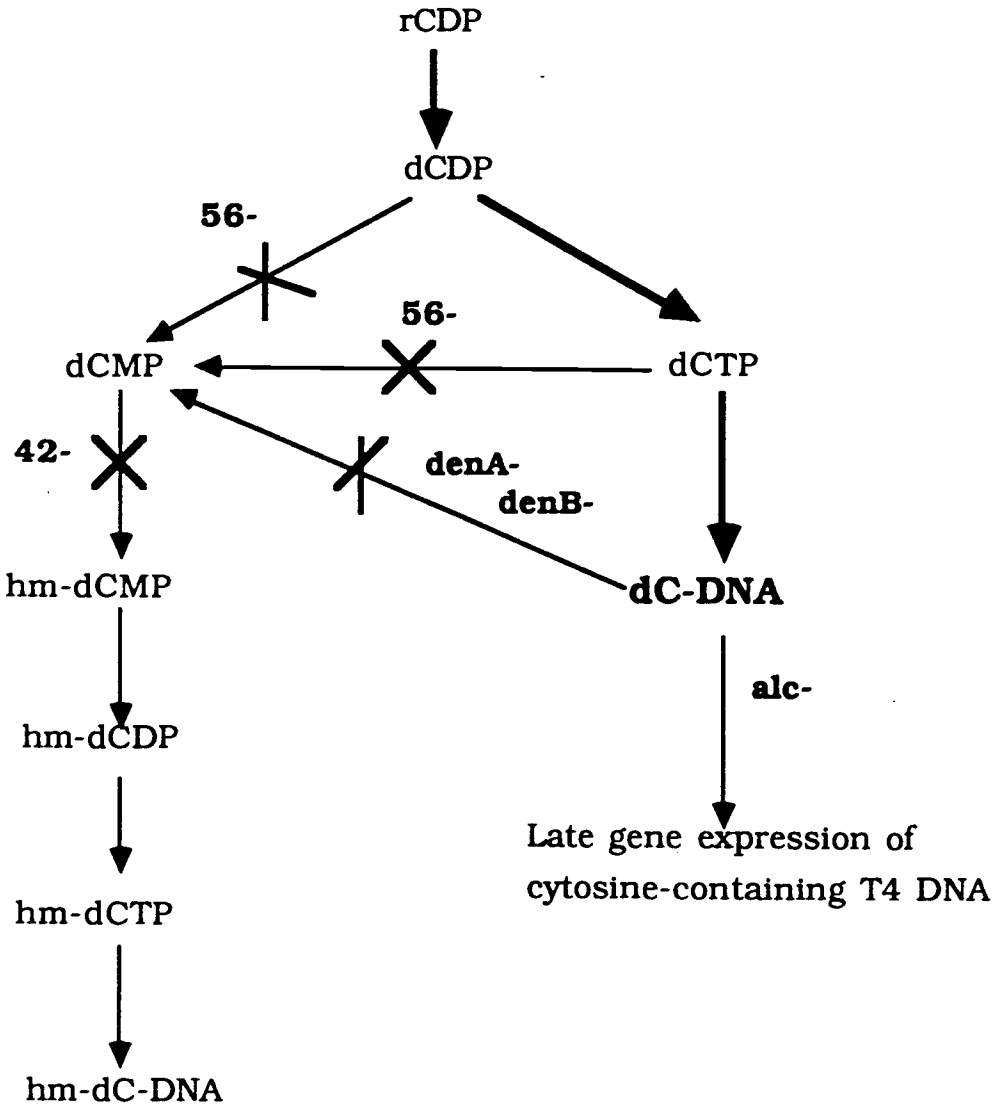


Figure I-6. Generation of viable bacteriophage T4 containing dC-DNA

of the two gene products prevents degradation of normal cytosine-containing DNA. The third mutation in BK536 is in gene 56 which codes for dCTPase. This mutation blocks conversion of dCTP and dCDP to dCMP so that accumulated dCTP can be incorporated into T4 DNA. In addition, the mutation in gene 56 also diminishes concentration of dCMP, which is the substrate of hydroxymethylase. The fourth mutation in BK536 is in gene 42, encoding dCMP hydroxymethylase. This mutation blocks the hydroxymethylation of dCMP so that no hm-dCTP is available to be incorporated into phage DNA. Mutants lacking in functions of genes 56, 42, *denA* and *denB* are able to synthesize cytosine-containing DNA; however, they cannot produce viable phage particles because the product of another gene, the *alc* gene, does not allow late gene transcription of cytosine-containing T4 DNA, and hence no late proteins are synthesized. Therefore, an additional mutation in the *alc* gene is essential for the production of viable phage particles assembled with cytosine-containing T4 DNA. BK536 actually achieves the production of cytosine-containing DNA by four effects: 1, enhancing accumulation of dCTP to be efficiently incorporated into T4 DNA; 2, preventing production of hydroxymethylated dCMP so that no hydroxymethyl dCTP is available to be incorporated into T4 DNA; 3, inactivating cytosine-containing DNA specific endonucleases in T4-infected *E. coli* cells; 4, permitting cytosine-containing DNA to serve as template for late gene transcription, so that viable phage can be assembled.

11. T4-induced Thymidylate Synthase and Dihydrofolate Reductase

Both thymidylate synthase and dihydrofolate reductase participate in deoxythymidylate biosynthesis. As shown in Figure I-5, thymidylate synthase transfers a methyl group from 5,10-methylene tetrahydrofolate to the pyrimidine ring of dUMP. In the same reaction, tetrahydrofolate is oxidized to dihydrofolate. Dihydrofolate reductase reduces dihydrofolate to tetrahydrofolate, which in turn carries another one-carbon group provided by serine, and keeps the reaction going. Dihydrofolate reductase not only supplies tetrahydrofolate for the thymidylate synthase-catalyzed reaction but is also involved in the biosynthesis of purine nucleotides and amino acids such as glycine and methionine. Because of its unique function, dihydrofolate reductase is a target enzyme for a number of antineoplastic, antiparasitic, immunosuppressant and antibacterial agents.

Thymidylate synthase as the phage-induced enzyme was first demonstrated by Flaks and Cohen (Flaks and Cohen, 1957; Flaks and Cohen, 1959). It was shown that its enzymatic activity was increased by severalfold after infection of *E. coli* B by T-even phage. The *td* gene, which codes for thymidylate synthase, was mapped very close to the *frd* gene, which codes for dihydrofolate reductase, on the T4 genetic map, as shown in Figure I-7. Thymidylate synthase from bacteriophage T4, like other known thymidylate synthases, is a homodimer with a subunit M_r of 32,000 (Belfort et al., 1983).

Dihydrofolate reductase, like thymidylate synthase, is a phage-

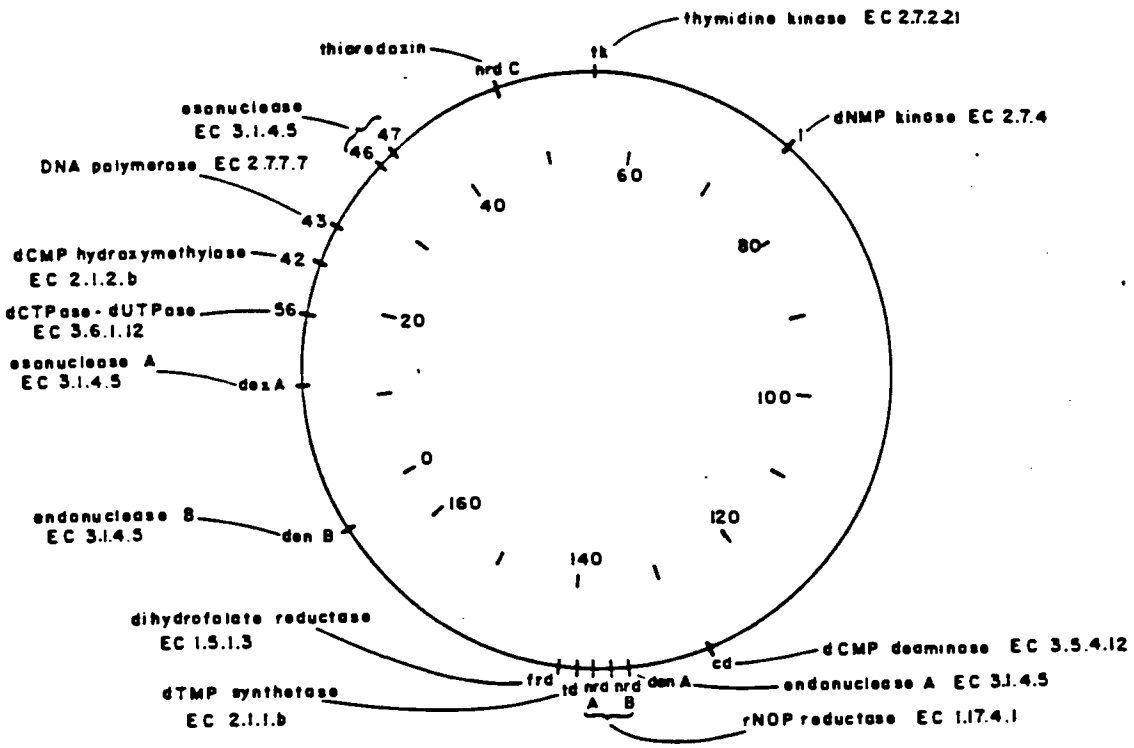


Figure I-7. Map positions of T4 genes encoding enzymes of DNA precursor biosynthesis. The numbers in the interior represent distance in kilobases from the rIIA/rIIB cistron divide (Mathews and Allen, 1983).

coded early enzyme and duplicates the preexisting host function. T-even phage-induced dihydrofolate reductase was first demonstrated by Mathews and Cohen (1963). The phage-induced enzyme was differentiated from host enzyme by its ammonium sulfate precipitation pattern, and its utilization of NADH as well as NADPH in the reaction. The concept of T-even phages encoding their own DHFR was further supported by isolation of bacteriophage *frd* mutants (Hall et al., 1967; Mathews, 1967). T4-induced dihydrofolate reductase is a dimer of 44,500 Daltons with a subunit molecular weight of 23,000 (Purohit et al., 1981).

12. T4 *td* Gene Encoding Thymidylate Synthase Is the First Structural Gene Found Containing an Intron in Procaryotic Organisms

Many protein-encoding genes in eucaryotic organisms are interrupted by one or more intervening sequences which are transcribed but not translated. Usually such noncoding sequence, introns, are excised posttranscriptionally, and the resulting coding sequences, exons, are ligated via the process of RNA splicing (Abelson, 1979). Although this process has been shown to be prevalent in eucaryotes, its possible occurrence in procaryotes was not considered until the discovery of the intron in the thymidylate synthase gene (*td*) of bacteriophage T4 (Chu et al., 1984). The presence of a 1016-base pair (bp) interruption separating the coding region for thymidylate synthase into two exons was revealed by comparing the amino acid

sequence determined for peptides derived from the purified enzyme protein with that deduced from the DNA sequence of the gene. The formation of mature mRNA of the *td* gene has been shown to be preceded by intron excision and exon ligation at the level of RNA in both *in vitro* (Chu et al., 1985) and *in vivo* (Belfort et al., 1985). Subsequent determination of the nucleotide sequence in the *td* intron has revealed that it possesses many structural features typical of eucaryotic class I introns (Cech, 1983; Michel et al., 1982; Waring and Davies, 1984). Furthermore, the *td* primary transcript has been shown to undergo self-splicing *in vitro* (Chu et al., 1985; Chu et al., 1987; and Chu et al., 1986) similar to that found for the *Tetrahymena thermophila* nuclear large rRNA (Cech et al., 1981; Zaug et al., 1983) and for many yeast and fungal mitochondrial pre-RNAs (Garriga and Lambowitz, 1984; Tabak et al., 1984). An internal guide sequence consisting of nine consecutive nucleotides located just downstream of the 5' splice site has been designated for the *td* intron (Chu et al., 1986). It is believed that this guide sequence, present in the intron segment of self-splicing pre-RNA, brings the 5' and 3' splice sites into proximity via specific base pairing for precise exon ligation (Waring et al., 1982).

Subsequent discovery of more T4 phage introns, namely that in the *nrdB* gene encoding the T4 ribonucleotide reductase small subunit (Sjoberg et al., 1986; Gott et al., 1986) and that in SRF 55 or sunY (Tomaschewski and Ruger, 1987; Shub et al., 1987), has confirmed that the *td* intron was not an isolated case. Furthermore, T4 *td*

intron-like sequences are present in the genome of T2 and T6 bacteriophages (Chu et al., 1987; Pedersen-Lane and Belfort, 1987), also interrupting the respective genes. For instance, T2 phage contains introns in its *td* gene as well as in its SRF 55, while T6 phage contains only a single intron in its *td* gene.

The function and origin of these introns in T-even phages are not clear. It has been suggested (Gott et al., 1986) that because the expression of *nrdB* requires guanosine and/or its 5'-phosphate, this mechanism could modulate the level of ribonucleotide reductase in phage-infected cells. It follows then that the splicing of group 1 introns in T4 phage, particularly that of the *nrdB* gene, would indirectly affect the relative size of RNA and DNA precursor pools. However, two observations argue against this speculation. One is the extremely low concentration (less than 0.5 μM) of guanosine required for autocatalytic splicing *in vitro* (Chu et al., 1987), and the other is the nonessentiality of the *nrd* genes (Mosig, 1983).

13. Structural Roles of the Phage-induced Dihydrofolate Reductase

In addition to its catalytic activity, it has been found that phage-induced dihydrofolate reductase is also a structural component of the T4 baseplate. Kozloff et al. (1970) showed that a low level of dihydrofolate reductase activity is present in purified tail plates, but it is not measurable in the phage baseplate of a T4 *frd* mutant, which is defective in the gene for the soluble enzyme. In addition, phage infectivity is neutralized by incubation of phage particles with reduced

nicotinamide adenine dinucleoside phosphate (NADPH), a cofactor of dihydrofolate reductase. The results described above not only suggested that phage-induced dihydrofolate reductase is the structural component of the phage tail plate, but also suggested that the structural protein is the same as the soluble enzyme. On the other hand, other results of Kozloff et al. (1970) suggested that the structural reductase is quite different from the soluble reductase. (1) NADPH inactivation is reversible by nicotinamide adenine dinucleoside phosphate ($NADP^+$), suggesting that reduction of the bound pteridine in situ is reversible, although it is impossible to demonstrate significant reversibility of the soluble enzyme in solution (Mathews and Sutherland, 1965). (2) NADPH can also inactivate the infectivity of a T4 *frd*1 mutant, which is unable to produce the soluble reductase. (3) Inactivation of T4D is more rapid at pH 5.0 than at pH 7.3, even though the soluble reductase has essentially no activity at pH 5 (Mathews, 1967).

To verify the identity and the origin of the structural dihydrofolate reductase, Mathews (1971) did some genetic studies which showed that both the soluble and structural reductase are the products of the same gene. The genetic study was done by constructing a hybrid T4 frd^{T6} that carries the *frd* gene from T6 in T4 genetic background. It was accomplished by crossing T4 dihydrofolate reductase mutant *frd* 2 (Mathews, 1967) with wild-type T6, several back crosses with T4 *frd* 2 mutant, and selection for dihydrofolate reductase-positive phenotype after each cross. The soluble enzyme from the hybrid

phage T4 *frd*^{T6} shows the properties of the T6 enzyme. For instance, dihydrofolate reductase from T4 *frd*^{T6} is more sensitive to incubation at 40 C than the enzyme from T4D . When the heat inactivation experiment was done with the hybrid T4 *frd*^{T6} phage along with the wild-type T4 and T6, the hybrid phage showed an inactivation kinetics similar to those of wild-type T6 but not T4. This result indicated that the *frd* gene does affect the heat stability of phage particles and that both soluble and structural dihydrofolate reductase are coded by the same gene. However, both wild-type T6 and the hybrid T4 *frd*^{T6} particles are heat inactivated at a slower rate than those of wild-type T4, even though the soluble T6 dihydrofolate reductase is more sensitive to heat than the T4 soluble enzyme. This unexpected result could be explained if that the properties of the enzyme are changed considerably when it is bound into the rigidly organized matrix of the tail plate.

Another piece of evidence supporting the structural function of T4 dihydrofolate reductase comes from studies of inactivation of phage infectivity by antiserum against purified dihydrofolate reductase (Mathews et al., 1973). It was shown that inactivation of T4D bacteriophage by the anti-DHFR is specifically due to antibodies against the antigenic determinants of this enzyme. The conclusion was further supported by the fact that all T4D strains were inactivated to the same extent, whereas other related phages were inactivated to a lesser extent, and unrelated phages were not inactivated at all. Unexpectedly, the hybrid phage T4 *frd*^{T6} and a *frd* amber mutant,

T4D *frd*11, were also rapidly inactivated by the antiserum. It is reasonable to explain the result by assuming that conformational changes during tail assembly induce the exposure of antigenic site in these closely related strains to equal those found in normal T4D.

Later on, more studies were carried out to support the conclusion that T4-induced soluble dihydrofolate reductase is the structural component of the T4 baseplate. In particular, more direct approaches have been taken to confirm the early discovery. Mosher and Mathews (1977) attempted to identify and quantitate the enzyme proteins in T4 tails by polyacrylamide gel electrophoresis analysis of labeled virion proteins, but the attempt was unsuccessful.

14. T4-induced Thymidylate Synthase Is also a Structural Component of Phage Baseplate

In 1973, Capco and Mathews first demonstrated that T4-induced thymidylate synthase is also a structural component of the phage particle (Capco et al., 1973; Capco and Mathews, 1973). The first evidence came from an experiment showing that antiserum against purified T4 thymidylate synthase can neutralize phage T4 infectivity, and no neutralization of bacteriophage T2 and T6 infectivity was observed, although this antiserum cross reacts with the soluble thymidylate synthase from these phage on double diffusion plates. A hybrid phage, T4 td^{T6}, was constructed by using a technique similar to that used for T4 frd^{T6} construction in the study. When a heat inactivation kinetic study was performed with the hybrid phage, it

showed a similar kinetics to bacteriophage T6 but not T4. Additional evidence supporting the notion is as follows: 1, incubation of purified thymidylate synthase with antiserum decreases the phage-neutralizing effect of the serum; 2, incubation of the antiserum with T4 phage destroys the ability of the antiserum to react with the purified thymidylate synthase. However, a paradox was raised when hybrid phage T4 td^{T6} was used to do the antiserum neutralization experiment. T4 td^{T6} was sensitive to thymidylate synthase antiserum to the same extent even though wild-type T6 is resist to the antiserum neutralization. The paradox can be explained that incorporation of the enzyme in a rigid baseplate structure can result in a conformational change in its structure, and lead to the change in reactivity towards antiserum against T4-induced thymidylate synthase.

15. Deletion Mutants in Gene 63-32 Region

It was observed that phage carrying a duplication of the rII region (Weil et al., 1965; Parma et al., 1972; Parma and Snyder, 1973; Symonds et al., 1972) often contains a deletion (Parma et al., 1972; Weil and Terzaghi, 1970). Homyk and Weil (1974) used this knowledge and isolated a number of deletion mutants in nonessential regions of the T4 genome. They crossed two rII deletion mutants, r1589 (Champe and Benzer, 1962) and r1236 (Barnett et al., 1967), with overlapping deletions in the rII region. Although the mutant r1589 is deleted in part of the rIIA and rIIB cistron but retains cistron

B activity, r1236 has a deletion in cistron B. The two mutants complement each other functionally, however, so no wild-type phage recombinants can be obtained by such crosses. Thus, the mutant phages with rII duplications were selected by their ability to form plaques on an *E. coli* lambda lysogen that restricts the growth of rII mutants. Phage particles with abnormal plaque morphology were selected as the recombinant phage with possible deletions.

Several deletion mutants in the nonessential regions between genes 39-56 and between 63-32 were isolated. The lengths and locations of the deletions were identified by heteroduplex mapping, and possible functions of the deleted genes were further analyzed by enzymatic assays.

The 63-32 region of T4 genome spans a cluster of early genes *frd*, *td*, *nrdA*, *nrdB* and *denA*, as shown in Figure I-8. T4 *nrdA* gene codes for the large subunit of T4-induced ribonucleotide reductase while *nrdB* gene codes for the small subunit of the enzyme. All the genes except denA duplicate pre-existing activities in host cells. Three of the deletion mutants in the 63-32 region have been studied extensively. These mutants, *del* 1, *del* 7, *del* 9 (Homyk and Weil, 1974), although viable, grow poorly in general, and they are restricted completely by several wild-type *E. coli* strains in the Cal. Tech. collection (Mosher et al., 1977). Those Cal. Tech. strains do not restrict either wild-type T4D or the parental mutant, r1589. These three deletion mutants lack thymidylate synthase and ribonucleotide reductase activities (Capco et al., 1973; Mosher and Mathews, 1977;

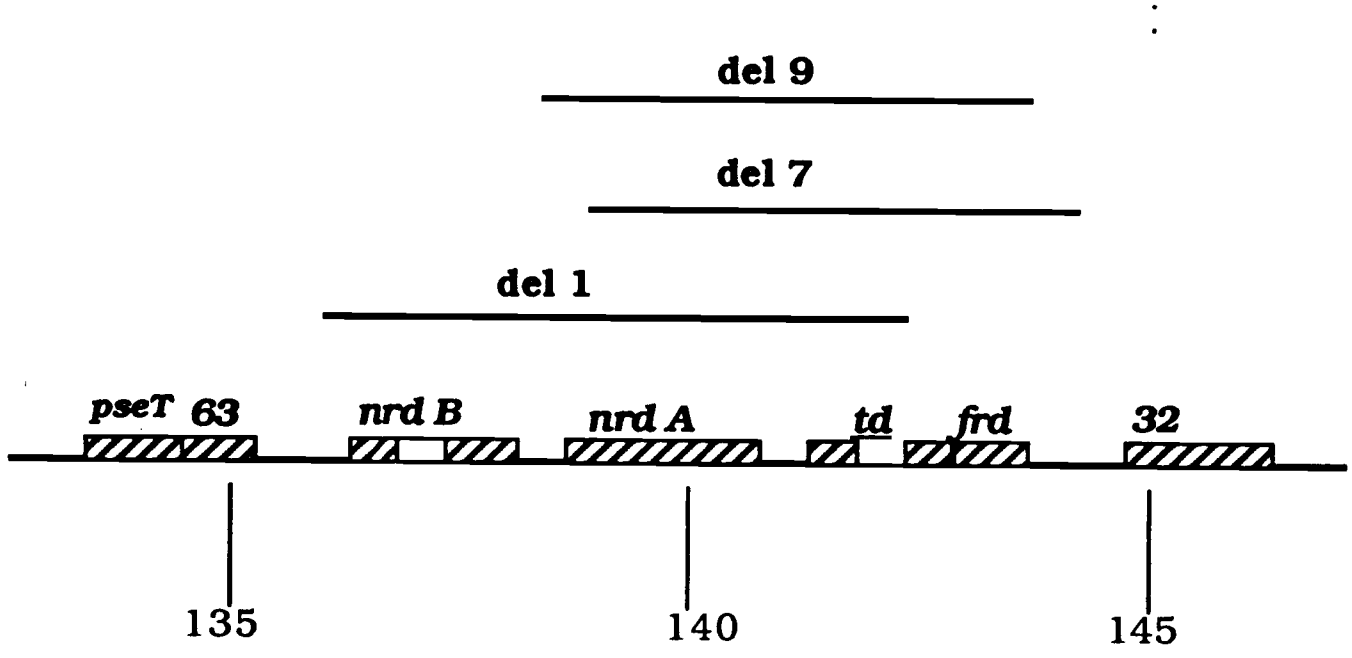


Figure I-8. Map of 63-32 region of T4 DNA showing deletions in *del 1, del 7 and del 9* mutants. The deletions were mapped by Homyk and Weil (1974) by heteroduplex mapping. The numbers indicate kilobase pairs from the rIIA/rIIB cistron divide.

Homyk and Weil, 1974; and Kozloff et al., 1977), and *del* 7 and *del* 9 are also deficient in dihydrofolate reductase activity (Mosher et al., 1977; Capco et al., 1973).

In studies of the structural role of T4-induced thymidylate synthase and dihydrofolate reductase, the three deletion mutants, *del* 1 *del* 7, and *del* 9, have been used as putative negative controls to confirm the previous discovery. The rationale for study with the deletion mutants is as follows. If the deletion mutants were indeed missing the structural genes for T4 thymidylate synthase and dihydrofolate reductase, they would be unable to produce phage-specific enzymes. Consequently, the mutant phage particles would lack both thymidylate synthase and dihydrofolate reductase in their baseplates. When antiserum inactivation experiments were done with thymidylate synthase antiserum and the deletion mutants (Capco and Mathews, 1973), surprisingly, the deletion mutants were inactivated by the antiserum at a rate similar to that of T4D. Phage neutralization experiments were also carried out with dihydrofolate reductase antiserum (Mosher et al., 1977). Again, the deletion mutants were even more readily inactivated by dihydrofolate reductase antiserum than was wild-type T4D. A possible explanation proposed for the unexpected result was that the *td* gene, encoding thymidylate synthase, and the *frd* gene, encoding dihydrofolate reductase, are not completely deleted in the mutants, so that the truncated proteins can still be produced and be incorporated into the tail baseplate during phage assembly. The experimental result supporting the explanation

was double immune diffusion (Capco and Mathews, 1973; Mosher et al., 1977). It was shown that a cell extract of *E. coli* infected by the deletion mutants contains cross-reacting materials to the antisera against thymidylate synthase and dihydrofolate reductase.

16. Present Work

The purpose of the present work was to clone and overexpress T4 gene 42, encoding dCMP hydroxymethylase. The cloning project includes the cloning of a mutant gene 42, converting the mutant gene back to the wild-type gene by site-directed mutagenesis and overexpression of the wild-type T4 gene 42. The expressed enzyme was tested by N-terminal amino acid sequencing and specific activity determination. To study interactions of the deoxyribonucleotide biosynthetic enzymes, the cloned dCMP hydroxymethylase was immobilized and used to carry out affinity chromatography. The enzyme purified from the cloned gene is also being used to study its catalytic mechanism and crystal structure. Polyclonal antibody was generated with the purified protein and has been used to study the deoxynucleotide biosynthesis complex in a variety of approaches such as column chromatography, immunoaffinity chromatography and cross linking.

For further study of the structural function of T4-encoded thymidylate synthase and dihydrofolate reductase, two deletion mutants, *del* 7 and *del* 9, were carefully characterized by the Southern blotting technique, as well as by immunological and enzyme

assays. The well characterized deletion mutants were used to further pursue the study of the structural functions of the TS and DHFR.

II Molecular Cloning and Expression of Bacteriophage T4 Gene 42 Coding for dCMP Hydroxymethylase

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1. Introduction

As mentioned in the General Introduction, significant amounts of deoxyribonucleotide biosynthetic enzymes are needed for the study of protein-protein interaction among the enzymes with *in vitro* systems. Furthermore, biochemical and biophysical studies of the enzymes also require availability of the cloned genes coding for the enzymes and overexpression of the gene products. The research described in this chapter is molecular cloning and overexpression of T4 gene 42 encoding dCMP hydroxymethylase.

2. Materials and Methods

1). Reagents

The restriction enzymes used for cloning were purchased from Bethesda Research Laboratories (BRL) and New England BioLabs, and used as specified by the vendors.

E. coli DNA polymerase I (large fragment) was from Worthington Biochemicals. T4 DNA ligase and bacterial alkaline phosphatase were purchased from BRL. Pancreatic DNase I and RNase were obtained from Sigma. Deoxyribonucleoside triphosphates of highest quality used for oligonucleotide-directed mutagenesis were from P-L Pharmacia, and dCMP used for dCMP hydroxymethylase assay was purchased from Sigma. [5-^{3}H]-dCMP used for tritium release assay was purchased from Schwarz/Mann, while [^{14}C]-formaldehyde used for ^{14}C dCMP hydroxymethylase assay was from New England Nuclear.

The 23-mer oligonucleotides used for site-directed mutagenesis were synthesized by Dr. R. McParland of the Center for Gene Research and Biotechnology, Oregon State University.

2). Bacterial Strains and Bacteriophage T4 Strains

Those strains used in the studies are listed in Table II-1.

3). Plasmid Vectors

Four plasmid vector systems were used during the cloning and expression of bacteriophage T4 gene 42. They are pUC19, pBSM13, pPL2388 and pT7 cloning systems. pUC19 used for the initial cloning

Table II-1. Bacterial and Bacteriophage T4 Strains.

E. coli strains	Genotype or phenotype	Source
B	wild type.	our collection
CR63	supD serine amber suppressor	our collection
B201	thy^- sup^-	our collection
RR 1	supE44 $recA^+$	our collection
JM 103	F' supE *del*(lac-pro)	our collection
JM 101	F' rec A^+ supE44 *del*(lac-proAB) lac^qZ-del M15	Strategene
JM 109	same as JM101 but $recA^-$	Strategene

T4 phage	genotype or phenotype	source
T4D	wild type	our collection
42amC87	$C87,42^-$	J. Wiberg
42amNG205	$NG205,42^-$	J. Wiberg
42amN122	$N122,42^-$	W. B. Wood
42amN55X5	$N55X5,42^-$	J. Wiberg
42am269X3	$269,42^-$	J. Wiberg
42tsLB-1	$LB\text{-}1,42^-$	W. B. Wood
42tsLB-3	$LB\text{-}3,42^-$	W. B. Wood

has been maintained in our laboratory for some times; pBSM13 used for site-directed mutagenesis was purchased from Strategene; pPL2833 expression system was obtained from Dr. Erik Remaut at University of Ghent; and the pT7 vector system used for overexpression of the gene was kindly supplied by Dr. S. Tabor at Harvard Medical School.

4). Compositions of the Media

These are listed in Table II-2.

5). Isolation of Plasmid DNA

a. Two methods used for small scale isolation of plasmid DNA were the boiling method (Holmes and Quigley, 1981) and the alkaline lysis method (Maniatis et al., 1982). The small scale plasmid isolation was frequently used for quick scanning of the recombinant plasmids for size.

b. Large scale isolation of plasmid DNA was carried out with a scaled up version of the alkaline lysis method.

6). Isolation of the Restriction Fragments

The first method used for isolation of restriction DNA fragments was electroelution. The DNA fragments were separated by electrophoresis on an agarose gel, and the DNA bands were identified by illumination with 300 nm ultraviolet light after staining the DNA

Table II-2. Compositions of the Media.

Media.	Composition. / Liter
Nutrient Broth.	8 g Nutrient broth; 5 g NaCl.
Hershey Soft Agar.	8 g Nutrient broth; 5g NaCl 7 g Bacto agar.
Nutrient Agar Plates.	1 Liter Nutrient broth; 15 g Bacto agar.
Luria-Bertani Broth.	10 g Bacto tryptone; 5 g yeast extract; 5 g NaCl; 1 g glucose.
M9 minimal medium	6 g Na_2HPO4; 3 g KH_2PO4; 0.5 g NaCl; 1 g NH_4Cl; 0.5 % glucose.
SM9	990 ml M9 medium; 10 ml 20% Casamino Acids.
Rich medium.	12 g Bacto tryptone; 4 ml glycerol; 24 g Bacto yeast extract; dissolved in 900 ml ddH_2O and autoclaved. Then 100 ml of sterilized 0.17 M KH_2PO4/0.72M K_2HPO4 buffer was added.

with ethidium bromide. A portion of the agarose gel containing the DNA fragment of interest was cut from the slab gel and placed in dialysis bags with approximately 1.5 ml 0.5 X electrophoresis buffer. The dialysis bags were clamped closed and placed in an electroelution apparatus, which was connected to a thermostatic cooling bath. The electroelution chamber was filled with 0.5 X TBE electrophoresis buffer to a level just above the DNA-containing dialysis bags, and the electroelution was carried out at 100 V constant voltage for overnight. Following electroelution, the eluted DNA was phenol extracted, and was collected by ethanol precipitation.

The second method used for isolation of restricted DNA fragments was the frozen phenol method. DNA fragments were separated, identified, and the agarose containing the DNA fragment of interest was cut from the gel in the same way as the one used in the electroelution method. The agarose was placed into a 1.5-ml Eppendorf tube and mashed with a plastic masher in the tube. Then it was mixed with an equal volume of phenol, and the mixture was vortexed for 5 minutes and kept at -80 C for 15 minutes. Following 15 minutes centrifugation in a microfuge, DNA in the aqueous phase was transferred to a new tube, phenol extracted and precipitated with 2.5 M ammonium acetate pH 7 and 2 volume 100% ethanol.

7). Transformation of DNA into *E. coli* Cells

One milliliter of *E. coli* cell culture was inoculated in 10 ml of fresh LB medium with the selection of antibiotics. The cells were

grown at 37 C for 1.5 - 2 hours, until a cell density of 2 X 10^8 cells/ml was reached. The culture was chilled on ice, and the cells were collected by centrifugation at 4 X 1000 g for 5 minutes. The cell pellet was resuspended in 5 ml of ice cold 50 mM $CaCl_2$, 10 mM Tris-Cl, pH 8, and was kept on ice for 30 minutes. The cells were then pelleted again by centrifugation, and resuspended in 1 ml of ice cold Tris-$CaCl_2$ solution. After 1 hour incubation on ice, 0.2 ml of the $CaCl_2$-treated cell suspension was mixed with 5 µl diluted DNA ligation mixture. The transformation reaction was allowed to proceed on ice for 30 minutes, and the mixture was heat-shocked at 42 C for 2 minutes. One milliliter of L broth was added and the transformed cell culture was incubated at 37 C for 1 hour. About 200 µl of the culture was spread on LB plates containing antibiotics for primary screening.

8). Molecular Cloning of the 1.8-kb T4 Fragment into pUC19 Vector

One microgram of the 8.9-kb Xho I T4 DNA fragment was digested with 10 units of Eco RI restriction enzyme at 37 C for 2 hours. The digestion product was phenol extracted and ethanol precipitated. DNA was collected by centrifugation at 4 C for 15 minutes in a microfuge, and the pellet was resuspended in 10 µl sterilized double distilled water. pUC19 vector DNA was linearized with Eco RI, and its ends were dephosphorylated with bacterial alkaline phosphatase. To set up a ligation reaction, the resuspended insert DNA was mixed with 100 ng of the vector DNA in 1 µl, along with 2µl of 10 X ligation

buffer, 0.5 units of DNA ligase in 1 µl, and 6 µl of H_2O. The ligation was carried out overnight at 12 C. Five microliters of this ligation mixture was used to transform *E. coli* JM 103 by the calcium chloride method as described earlier. The transformed cells were spread on LB agar plates, containing 50 µg/ml ampicillin, 40 µg/ml IPTG, and 100 µg/ml X-gal, for primary screening. The plates were incubated at 37 C overnight. Next day, the white colonies were selected and grown, and the recombinant plasmids were isolated for further characterization.

9). Subcloning of the 1.8-kb DNA Fragment Isolated from pUC1910 into other Plasmid Vector Systems

Recombinant plasmid pUC1910 was digested with Eco RI, and the 1.8-kb Eco RI fragment was isolated by either electroelution or the frozen phenol method as described above. One hundred nanograms of the isolated DNA was ligated with 50 ng of vector DNA, which was linearized with the same restriction enzyme and dephosphorylated at the ends. The ligation reaction was carried out at 12 C overnight, and 5 µl of the reaction mixture was used to transform *E. coli* cells by the calcium chloride method. The transformed cells were selected by plating on drug(s)-containing LB agar plates.

10). Preparation of Cell Cultures

a. T4-infected *E. coli* cell culture

A culture of *E. coli* was grown at 37, with aeration to a cell density of 3×10^8 cells/ ml in nutrient broth. After addition of L-tryptophan

to 20 μg/ml, the cells were infected with T4 phage at a multiplicity of 5-10. The cells were harvested at the desired times by centrifugation at 4 X 1000 g for 10 minutes. The cell pellet was stored at -80, then desired.

b. Cell culture of *E. coli K38* containing pT7 recombinant plasmid and the helper plasmid pGP1-2

Cells containing both pGP1-2 and the pT7 recombinant plasmid were grown in enriched medium with 50 μg/ml ampicillin and kanamycin at 30 C. At A_{590} = 1.5, the cells were induced by shifting the temperature to 42 C for 30 minutes. Then rifampicin was added to a final concentration of 100 μg/ml, and the temperature was lowered to 37 C. The cells were grown at 37 C for an additional 2 hours and harvested by centrifugation at 4 X 1000 g for 10 minutes. The cell pellet was stored at -80 C.

11). Preparation of Crude Cell Extract for Enzyme Assay and SDS Polyacrylamide Gel Electrophoresis

Cells from 100 ml of culture were thawed and resuspended in 2 ml of M9 minimal medium. The cell suspension was sonicated at setting 6 of an Utrasonics Sonicator, model W-10. The cells were disrupted by 4 bursts, each of 30 seconds duration. The cell suspension was kept on ice during sonication. The translucent disrupted cell suspension was centrifuged at 10,000 x g for 30 minutes and the supernatant was used as enzyme source for enzyme

assays and SDS gel electrophoresis.

12) Protein Concentration Determination

Protein concentrations were determined by the Coomassie Blue binding assay (Peterson, 1983). Dye stock solution was made by dissolving 1 g of Coomassie Brilliant Blue G-250 (Eastman Kodak) in 200 ml of 88% phosphoric acid and 100 ml of 95% ethanol. To make 1 X dye solution, 100 ml of the stock solution was diluted to 400 ml with water and filtered through Whatman No. 1 filter paper. 10 µl of protein sample or protein standard (BSA) was added to 1.0 ml of water followed by 1.5 ml of filtered dye solution. The absorbance (595nm) of the sample was read after a 5 to 10 minute incubation at room temperature.

13). Deoxycytidylate Hydroxymethylase Assay

a. Assay with [^{14}C]-formaldehyde

dCMP hydroxymethylase was assayed as described by Pizer and Cohen (1962). The reaction mixtures contained 0.02 ml of 1 M potassium phosphate buffer, pH 7.0, 0.03 ml of 0.083 M [^{14}C]-formaldehyde (0.1 mCi/mmole), 0.10 ml of 0.025 M dCMP, 0.25 ml of 0.002 M L-tetrahydrofolate in 0.1 M Tris-HCl, pH 7.4, 0.2 M mercaptoethanol, and enzyme in 0.1 ml. The mixtures were incubated at 37 C for the desired length of times and reactions were terminated by adding 0.5 ml of 10% TCA. Mixtures were centrifuged in a microfuge to pellet TCA-insoluble material and the supernatant fluid

was removed. The pellet was then washed with 1 ml of 5% TCA, and centrifuged again. The radioactive hydroxymethyl-dCMP in the supernatant was purified by polyethyleneimine-cellulose thin layer chromatography as described by Randerath and Randerath (1967).

b. ^3H release assay

^3H release assay was performed essentially as described by Yeh and Greenberg (1967). The reaction mixtures contained 100 µl 2 x reaction buffer (60 mM KH_2PO_4, pH 4, 29.4 mM 2-mercaptoethanol, 2.56 mM tetrahydrofolate, 2 mM Na_3EDTA, and 2 x 10^{-7} M fluoro-dUMP), enzyme in 50 µl, and 30 µl H_2O. The reaction was initiated by adding 20 µl of 10 x substrate, 30 mM [5-^3H]-dCMP, and incubated at 37 C for a period of time. The reaction was terminated with equal volume of stop solution (15% activated charcoal in 4% PCA), then it was vortexed and spun in a microfuge to pellet down the free nucleotide adsorbed to charcoal. The radioactivity of the [^3H]-H_2O generated in the reaction in the supernatant was measured in a scintillation counter.

14). Site-directed Mutagenesis

a. Preparation of single-stranded DNA

A 30-ml culture of pBSM13-42 recombinant in JM101 was grown in LB medium (the recombinant should have been selected on an ampicillin plate previously). Once the cell density O. D.A_{600} was equal to 0.3, cells were harvested by centrifugation at 5 x 1000 g for 10 minutes at room temperature. The cell pellet was resuspended in

another 30 ml of fresh LB medium, and the cells were superinfected by 1.5 x 10^{11} VCS-M13 helper phages. The superinfected cell culture was grown for 6 hours at 37 C and shaken vigorously. Then cells in the culture were pelleted by centrifugation, and the hybrid phages in the supernatant were transferred to a clean tube and precipitated with 4% polyethylene glycol and 0.5 M NaCl for 15 minutes. The precipitated phages were collected by centrifugation at 15 x 1000 g in room temperature for 15 minutes. The phage pellet was resuspended in 2.5 ml of TE buffer (0.1 M Tris-Cl pH 8; 1 mM EDTA). Phage DNA was isolated by three extractions with phenol: chloroform (1:1) and two extractions with chloroform. Then 0.5 volumes of 7.5 M ammonium acetate, pH 7, and 2.5 volumes of 100% ethanol were added, and the tube was placed at -80 for 30 minutes. The DNA was pelleted by a 15-min centrifugation at 15 x 1000 g, washed with 0.5 ml of 70% ethanol, dried in vacuo, and resuspended in 500 µl of TE buffer.

b. Phosphorylation of the oligonucleotide

200 pmol of oligonucleotide was mixed with 2 µl of 10 x kinase buffer (1 M Tris-HCl, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, pH 8.3), 1 µl of 10 mM rATP, and 4 units of T4 polynucleotide kinase in a total volume of 20 µl. The reaction was carried out in a 1.5-ml Eppendorf tube incubated at 37 C for 1 hour and was terminated by heating at 65 C for 10 minutes.

c. Oligonucleotide-directed mutagenesis

Annealing reaction: 0.5 pmol of single-stranded template DNA was mixed with 10 pmol of 5' phosphorylated oligonucleotide and 1 µl of 10 x annealing reaction buffer (0.2 M Tris-HCl, 0.1 M $MgCl_2$, 0.5 M NaCl, 0.01 M dithiothreitol pH 7.5) in a total volume of 10 µl. The reaction mixture was heated at 55 C for 5 min, then placed at room temperature (23 C) for 5 minutes. During the annealing reaction, the enzyme/nucleotide solution was prepared by addition of the following components: 1 µl of 10 x extension/ligation buffer (0.2 M Tris-HCl, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, pH 7.5), 4 µl of 2 mM dNTPs, 1 µl of 10 mM rATP, 3 units of T4 DNA ligase, 2 units of *E. coli* DNA pol I (large fragment), and H_2O to 10 µl. This was kept on ice until use.

Extension and ligation: After 5 minutes at room temperature, 10 µl of the enzyme/nucleotide solution were added to the annealed DNA. The contents were mixed, then the tube was placed at 15 C for 6-12 hours.

Transformation: The reaction sample was diluted 20 x, 100 x, and 500 x with TE buffer, and 1 µl and 5 µl of each dilution was used to transform $CaCl_2$-treated *E. coli* B cells.

15). Purification of dCMP Hydroxymethylase from K38 *E.coli* Cells Containing Cloned Gene 42

The protocol used for the purification of T4 dCMP hydroxymethylase was nearly the same as the one for the T6 enzyme purification by Mathews et al. (1964). One change was made in the T4

dCMP hydroxymethylase purification when the enzyme was fractionated with a hydroxylapatite column. The enzyme bound to the hydrxylapatite column was eluted with a single buffer (0.12 M KPO_4, pH 6.5) instead of a linear salt gradient as used in T6 enzyme purification.

16). SDS Polyacrylamide Gel Electrophoresis

SDS PAGE was used frequently for protein analysis in the study. Compositions of the running gels and their stacking gel were listed in Table II-3. Size, concentration, and thickness of the gels were determined based on the size of the protein samples and the purposes of the experiments. For instance, a gel with high concentration was used to analyze proteins in the low range of the molecular weight, while a gel with low concentration was needed for Western blotting. Most of the gels were run at constant voltage, starting with 25 mA in current, and stopped when the phenol blue dye reached the bottom of the gels.

17). Western Blotting

The proteins to be probed were resolved on a low percent SDS polyacrylamide gel. Then the proteins were transferred to a nitrocellulose sheet with an electroblotter at 200-400 milliampere constant current for 4 hours to overnight. Transfer buffer was composed of 0.15 M Tris-HCl, 1.15 M glycine, pH 8.3. Sites on the nitrocellulose that do not contain proteins were blocked by one hour

Table II-3. Compositions of SDS PAGE running gels and stacking gel.

Gels	Running Gels				Stacking Gels
Conc. of the gels.	10%	12%	12.5%	15%	4%
Solutions					
Water	4.02 ml	3.35 ml	3.17 ml	2.4 ml	6.1 ml
4 X buffer	2.5 ml	2..5 ml	2.5 ml	2.5 ml	2.5 ml
30% Acryl -amide.	3.33 ml	4.0 ml	4.17 ml	5.0 ml	1.33 ml
10% SDS	100 µl	100 µl	100 µl	100 µl	100 µl
10% NH4-persulfate.	50 µl	50 µl	50 µl	50 µl	50 µl
Temed	10 µl	10 µl	10 µl	10 µl	10 µl

incubation in Tween buffer (0.5% Tween 20 in PBS) plus 1% gelatin. The nitrocellulose was then incubated with primary antibody diluted into 20 ml Tween buffer, and the incubation was carried out with agitation for 2 hours at room temperature. To remove the unbound antibody, the nitrocellulose was washed three time for 5 minutes each with Tween buffer. The secondary immune reaction was carried out by incubating the nitrocellulose with alkaline phosphatase-conjugated anti-rabbit antibody, which was diluted 1000-fold into Tween buffer, for 1 hour at room temperature. The nonspecific bound secondary antibody was removed by three times washing with Tween buffer for 15 minutes each. The final wash was done with 0.1 M ethanolamine-HCl, pH 9.6, and the nitrocellulose was stained with 10 ml of the following mixture: 9 ml of 0.1 M ethanolamine-HCl pH 9.6, 0.2 ml of 0.2 M $MgCl_2$, 0.15 ml of 0.4% BCIF (bis-chloroindophenol phosphate) prepared in 2:1 methanol-acetone; and 1 ml of freshly prepared 0.1% nitro blue tetrazolium.

3. Results

1). Cloning of a 1.8-kb Eco RI DNA Fragment of Bacteriophage T4 into pUC19 Vector

Because of the modification of wild-type T4 DNA and its resistance to digestion by most restriction enzymes, it is very difficult to clone T4 genes from modified phage DNA. A mutant T4 strain, BK536, was generated to overcome the difficulty (Kutter and Snyder, 1983). As described in the introduction, the mutant strain contains mutations in the genes encoding endonuclease II and IV, dCTPase, dCMP hydroxymethylase and alc, and it can generate unmodified cytosine-containing DNA for the cloning purposes. To clone T4 gene 42, encoding dCMP hydroxymethylase, I used a T4 DNA fragment from the mutant phage. Since gene 42 is mutant in BK536, I have to clone an amber mutant gene first and try to find a way to have the mutant gene produce wild-type enzyme.

I started the cloning project with an 8.9-kb Xho I DNA fragment from the mutant T4 strain BK536. Figure II-1 shows the restriction map of the DNA fragment. It contains five Eco RI restriction sites. Gene 42 is located in a 1.8-kb Eco RI fragment based on the restriction map. The plasmid vector selected for the initial cloning was pUC19. Several characteristics of the plasmid make it a widely used cloning vector. First, it has a high copy number in *E. coli* cells, so that the plasmid DNA can be obtained in large quantity from a small amount of cells. Second, it is only 2.8-kb in size. This facilitates

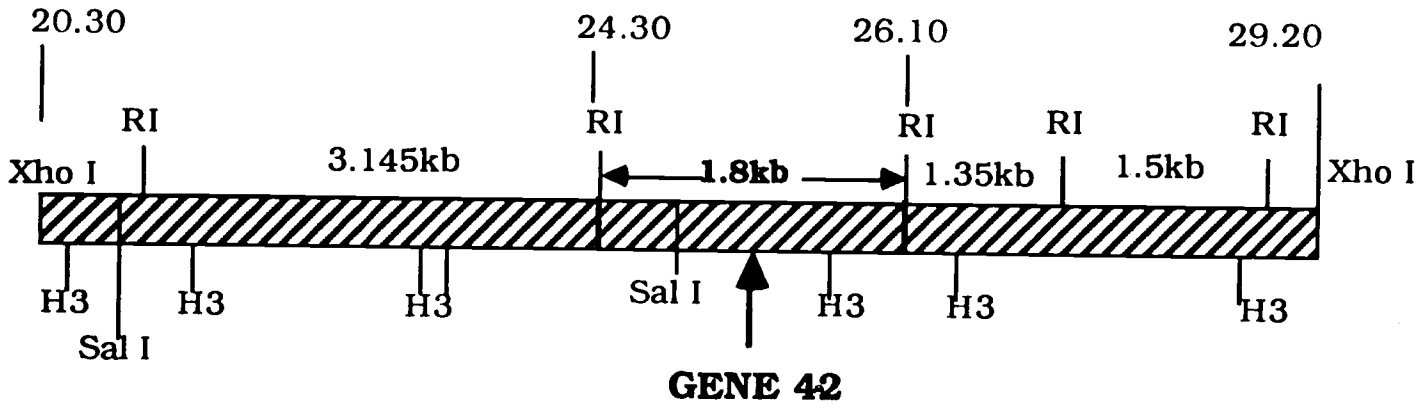


Figure II-1. Restriction map of the 8.9-kb XhoI DNA fragment of T4 phage, from which the 1.8-kb EcoRI fragment was cloned. The upper numbers indicate the distance in kb from the rIIA/rIIB cistron divide.

cloning a large piece foreign DNA without excessive difficulty in transformation. Third, due to the the fact that the plasmid contains a *lac* promoter, as well as part of the *lacZ* gene, the pUC19/foreign gene recombinant plasmid can be selected with IPTG and X-gal, which are the lac promoter inducer and ß-galactotransferase indicator, respectively. Furthermore, the cloned foreign gene can be controlled and expressed through the lac promoter.

The 8.9-kb XhoI DNA fragment was digested with Eco RI restriction enzyme, and the digestion product was ligated to pUC19 vector, which was linearized with the same restriction enzyme and dephosphorylated with bacterial alkaline phosphatase. The ligation reaction was transformed into $CaCl_2$-treated *E. coli* JM 103 cells, and the recombinant clones were selected by plating the cells on IPTG and X-gal-containing agar plates. Recombinant plasmids were isolated from JM103 cells and examined on agarose gel after electrophoresis and ethidium bromide staining. Figure II-2 shows that the recombinant plasmids isolated contain different inserts. To select the recombinant plasmids which contain the right inserts, the inserts were dropped by Eco RI digestion, and the sizes of the inserts were measured on agarose gel with standard size marker. Figure II-3 shows that both clone 13 and 10 contain an insert of 1.8-kb in size. Based on the restriction map of the 1.8-kb fragment, there is a Hind III restriction site near the 5' end of the fragment while there is a Sal I site near the 3' end of the fragment. The two restriction sites were used as references to further examine the clones 13 and 10 and to determine the orientations of the insertions. Clone 7 and 10 are

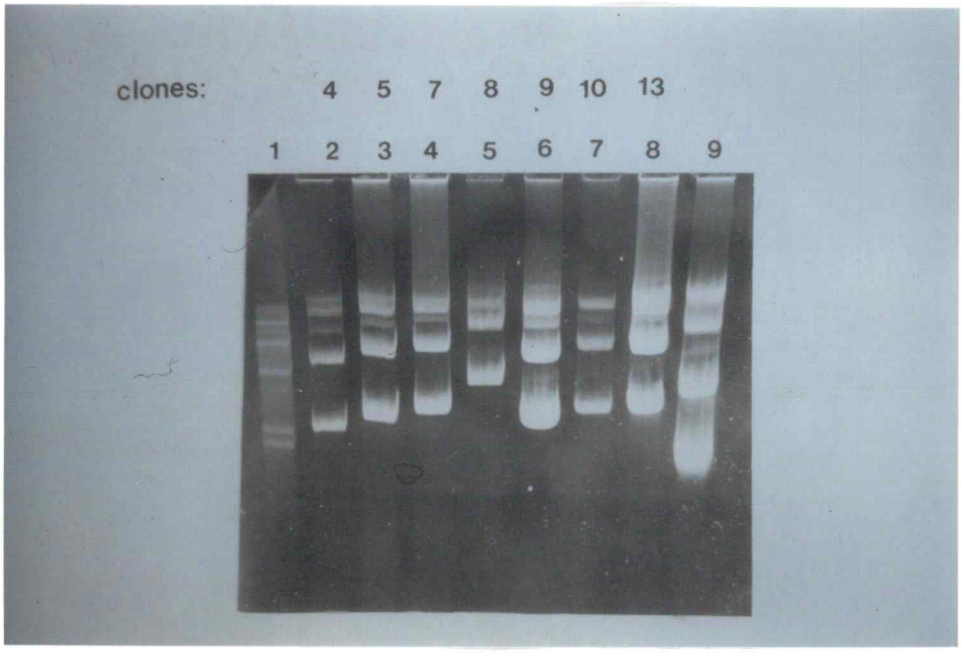


Figure II-2. A DNA agarose gel showing the recombinant plasmids resulted from the cloning of Eco RI digest of the 8.9-kb Xho I fragment into pUC19. Lane1, Hind III-digested DNA; lane 2 to 8, seven recombinant plasmids; and lane 9, pUC19 vector used for the cloning.

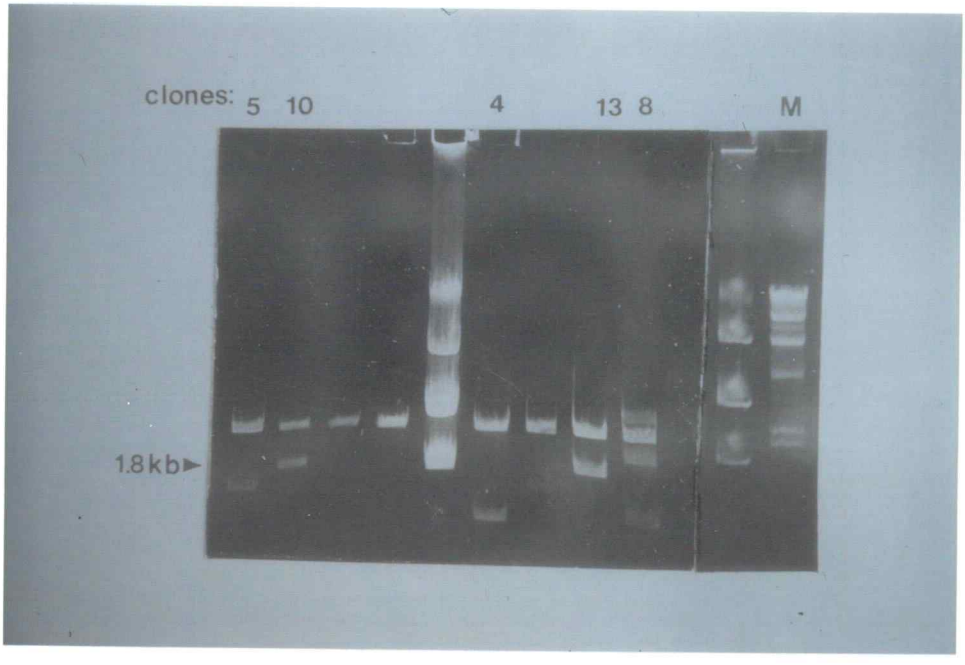


Figure II-3. Eco RI digests of the recombinant plasmids shown in Figure II-2. The sizes of the inserts dropped in the digestion were measured with the marker, and are identified by the arrows.

actually identical clones, and both of them contain the 1.8-kb DNA fragment as shown in Figure II-4 .

2). Genetic and Biochemical Tests of the Cloned 1.8-kb Eco RI Fragment for Its Gene 42 Activity

To test if the cloned 1.8-kb DNA fragment contains T4 gene 42, marker rescue was performed with several gene 42 mutant strains. Marker rescue as a genetic test was done by infecting the cloned 1.8-kb DNA-containing cell with a T4 gene 42 temperature-sensitive mutant or mutant strain bearing amber mutations at different loci in gene 42. If the cloned 1.8-kb DNA fragment does contain gene 42 sequence, the mutation in the infecting phage would be rescued by homologous recombination with the cloned sequence. The rescued temperature sensitive mutant phage, therefore, could grow at the restrictive temperature, and the rescued amber mutant phage could grow on sup$^-$ *E. coli* cells. The T4 gene 42 mutants used for marker rescue and their mutation loci are shown in Figure II-5. JM103 cells containing cloned gene 42 were grown to late exponential phase and seeded on nutrient agar plates with Hershey soft agar. The seeded cells were infected with 10^5 mutant phages in the small volume, and the infection was allowed to proceed overnight at a desired temperature. As expected, most of the gene 42 mutants were rescued by the cloned 1.8-kb DNA fragment except NG205. A possible explanation for NG205 not being rescued is that its mutation locus is so close to the mutation locus of the cloned mutant gene 42 that the

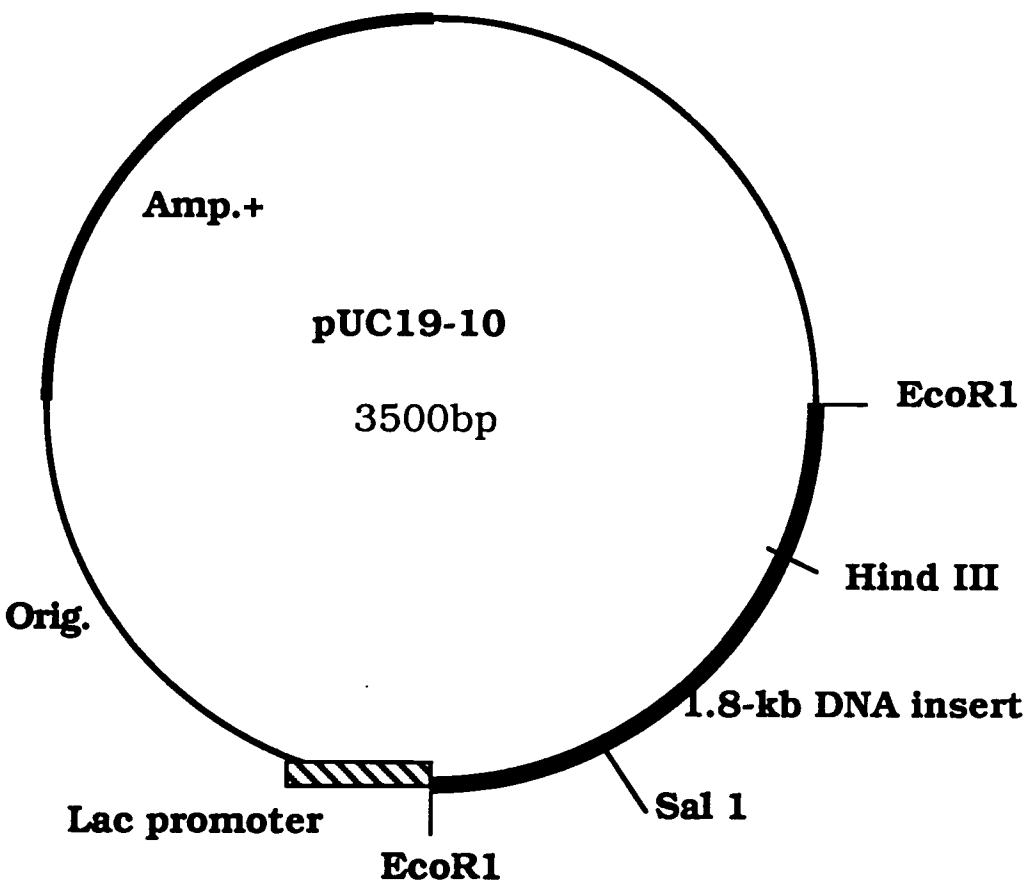
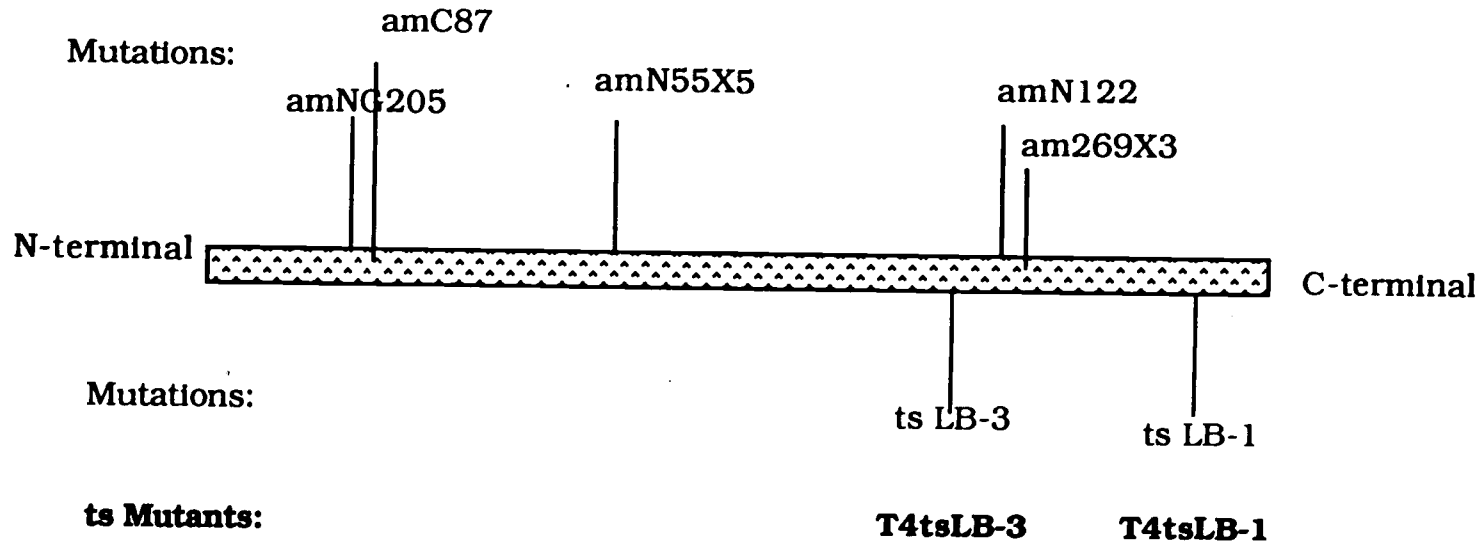


Figure II-4. Restriction map of the recombinant plasmid pUC19-10. Heavy line designates the 1.8-kb EcoRI insert.

amber Mutants: T4amNG205, T4amN55X5, T4am122, and T4am269X3.



T4 Gene 42

Figure II-5. T4 gene 42 mutants used for marker rescue and the locations of the mutations that the mutants bearing.

homologous recombination between the two mutant gene is rare.

Even though marker rescue can show the existence of gene 42 in the 1.8-kb DNA fragment, it cannot tell if the fragment contains the whole gene. This question only can be answered by biochemical assay of the enzymatic activity. However, we cannot assay the enzyme activity of the cloned gene 42 in a wild-type *E. coli* strain because of the amber mutation in the gene. One way to overcome the problem is to transform the putative mutant gene 42 into an amber suppressor cell, in which the amber mutation can be masked. In an amber suppressor cell, mutant tRNA with an amber anticodon can bring an amino acid to the amber codon of a translating mRNA. As a result, translation of an amber mutant mRNA will not be stopped at the amber codon, and a full length protein can be produced instead of a truncated and nonfunctional peptide. The amber suppressor *E. coli* strain we used was a serine suppressor cell, CR63. The mutant tRNA in the cell can take a serine amino acid to the amber codon during translation and mask the amber mutation. The cloned 1.8-kb DNA fragment was transformed into CR63 cells, and a cell extract was prepared to test its hydroxymethylase activity. As shown in Figure II-6, the cloned 1.8-kb Eco RI fragment expresses hydroxymethylase activity at a threefold higher level than T4-infected CR63 cell.

3). Strategies to Make the Cloned Mutant Gene 42 to Produce Wild-type Active Enzyme

Once we were assured that the cloned 1.8-kb DNA fragment

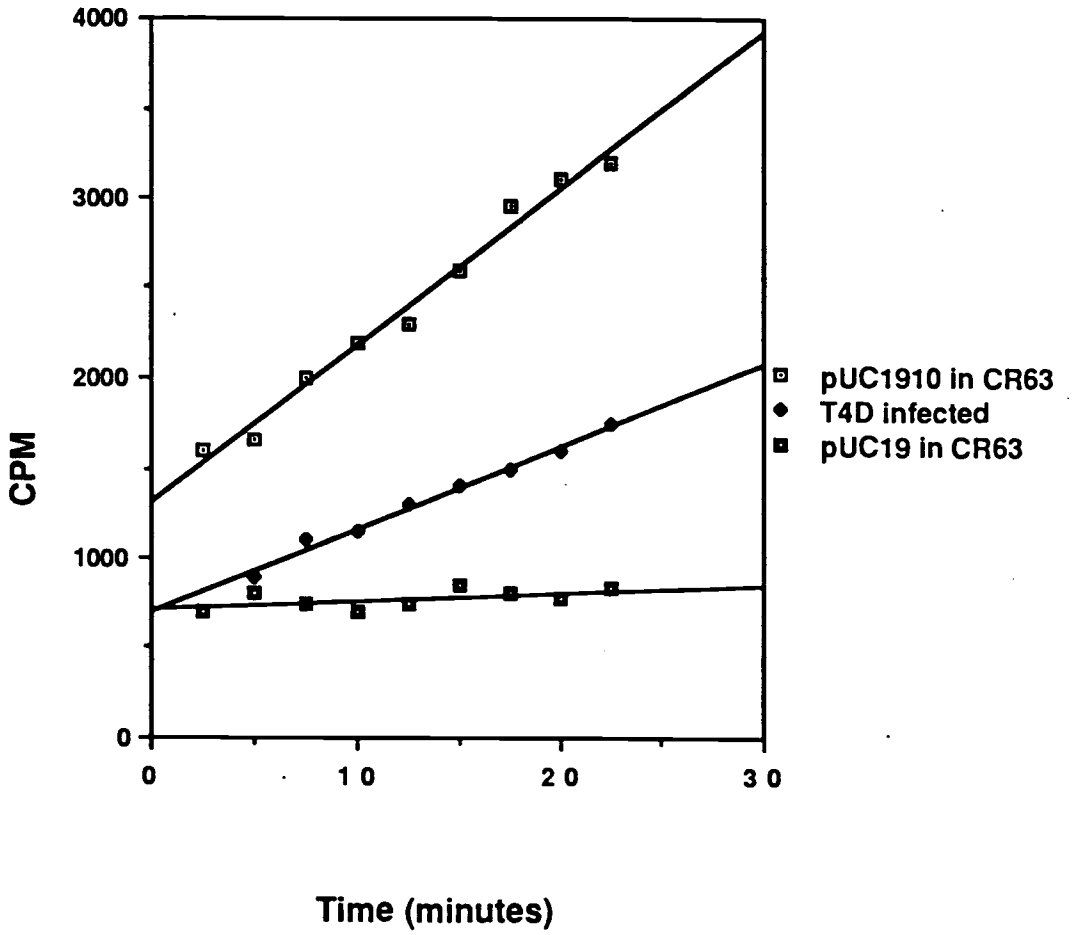


Figure II-6. Activity of dCMP hydroxymethylase expressed from the cloned mutant gene 42 in CR63 amber suppressor cell. T4-infected cell extract and pUC19-containing cell extract were used as controls in the enzyme assays.

contains the whole mutant dCMP hydroxymethylase gene, we tried to find a way to obtain native enzyme from the mutant gene. At the time we cloned mutant T4 gene 42, Ruger's laboratory in West Germany had been doing T4 DNA sequencing after shot-gun cloning of T4 DNA. They sequenced the region of gene 42, but were unable to obtain a DNA fragment containing the whole gene 42 for expression purpose. We agreed to collaborate by using their DNA sequence information to express wild-type enzyme from our cloned mutant gene 42. There are two reasonable ways to produce wild-type dCMP hydroxymethylase from the mutant gene. One way is at the DNA level through backward mutagenesis. Another way, a simpler way, is at the protein level by transforming the cloned mutant gene 42 into an amber suppressor cell as has been done for testing the biochemical activity of the mutant gene. Based on the nucleotide sequence data provided by Norbert Lamm from Ruger's laboratory, the amber codon in the mutant gene 42 was a glutamine codon before the mutation occurred. A cytosine to thymidine transition converts the glutamine codon into an amber stop codon in the mutant gene. To obtain wild-type native enzyme, we cannot use suppressor cell CR63 to rescue the mutation in gene 42 because CR63 is a serine suppressor cell not a glutamine suppressor cell. In other words, a serine amino acid, instead of a glutamine amino acid which is the native residue at that position, will be incorporated into the amber codon position during translation. Therefore, we had to find a glutamine suppressor cell for the production of wild-type enzyme from the mutant gene, as schematic demonstrated in Figure

II-7. There are sveral *E. coli* strains which are glutamine suppressor cells, such as HB101, RRI and JM cells. We collected the supE suppressor cells and tested their amber suppression capacity. The test was done in two ways. First, the supE cells were infected by gene 42 amber mutant T4 phages, and the size of the plaques was used as index to measure the suppression ability of the cell. The alternative way to test the cells' suppression capacity was to measure the hydroxymethylase enzyme activity after transforming the cloned T4 amber gene 42 into the suppressor cells. Unfortunately, all the supE amber suppressor cells we collected do not have high enough capacity to suppress the amber mutation of T4 gene 42, as shown in the plaque size measurement, and the enzymatic assay shown in Table II-4.

In order to further extend the approach, I obtained some more glutamine suppressor strains from *E. coli* Genetic Stock Center. The same tests were performed with those suppressor cells. Again, none of the strains have high enough suppression capacity to supply the production of the native functional enzyme.

The second approach, site-directed mutagenesis, was depended upon for producing T4 wild-type dCMP hydroxymethylase from the cloned mutant gene. Site-directed mutagenesis has been widely used to introduce specific mutations into a gene. It is a very powerful tool to study the function of a specific amino acid of a protein. To do those studies, normally a change of a single nucleotide at DNA level will lead to an amino acid substitution of the wild-type protein. For our purpose, we actually did a backward site-directed mutagenesis. In other words, we converted a mutant gene to a wild-type so that it

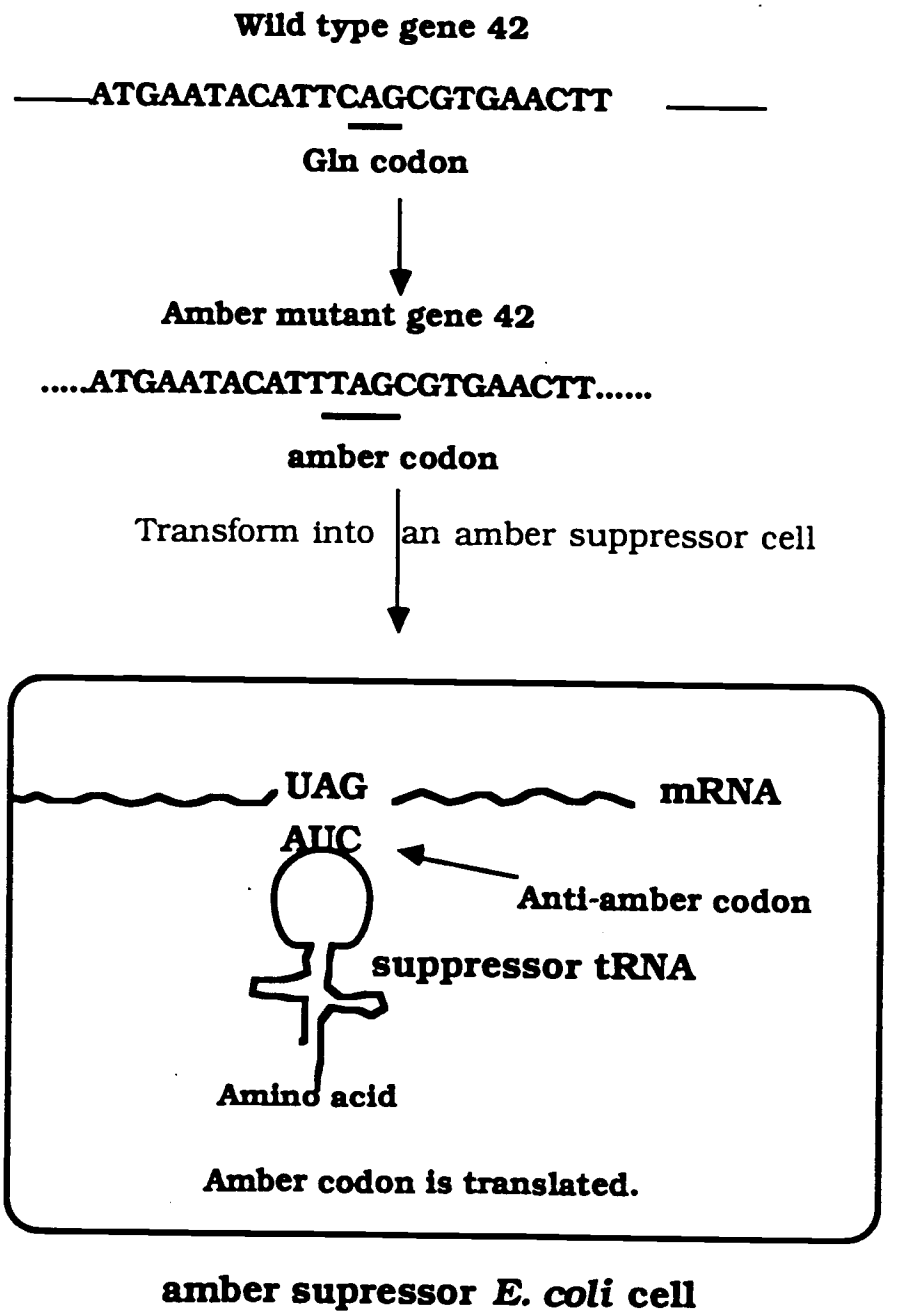


Figure II-7. Schematic representation of the suppression of T4 gene 42 amber mutation in an amber suppressor *E. coli* strain.

Table II-4. Activity of dCMP hydroxymethylase expressed from cloned mutant gene 42 in amber suppressor E. coli cell.

Activities (cpm) / cells	Time (minutes) 0	5	10	15	20
pUC1910 in CR63	546	1640	2170	2610	3075
pUC1910 in C600	525	548	615	538	574
pUC1910 in RRI	530	514	557	561	623
pUC1910 in JM103	574	552	682	575	546

could produce native enzyme.

As shown in Figure II-8, a single-stranded form of a template DNA is preferred in site-directed mutagenesis. The vector that I chose to generate single-stranded form of gene 42 is pBSM13. pBSM13 is a hybrid of pUC vector and M13 phage. The pUC part of the vector provides all the special features of the pUC plasmid such as lacZ gene expression and selection system, ampicillin resistance and the high copy number of the plasmid. In addition, T3 and T7 promoters are inserted in the sites near the lac promoter so that the cloned gene can be expressed in an *in vitro* system. The M13 part of pBSM13 provides the information to let the vector DNA be synthesized in a single-stranded form, packaged and extruded out of the cell with the assistance of a helper phage. The vector was selected for our particular usage because it not only can provide single-stranded DNA, but it also can be very stable as double-stranded DNA in the host cell. DNA as double-stranded form in the host cell is very important for the selection of wild-type gene 42 by marker rescue after site-directed mutagenesis.

The 1.8-kb Eco RI DNA fragment containing mutant gene 42 was excised from pUC1910 with Eco RI restriction enzyme and subcloned into pBSM13$^-$. The orientation of the insertion was determined by physical mapping with two restriction enzymes, Hind III and Sal I. The recombinant plasmid was named as pBSM13-42 and its restriction map is shown in Figure II-9. To prepare single-stranded DNA for site-directed mutagenesis, JM101 *E. coli* cells bearing

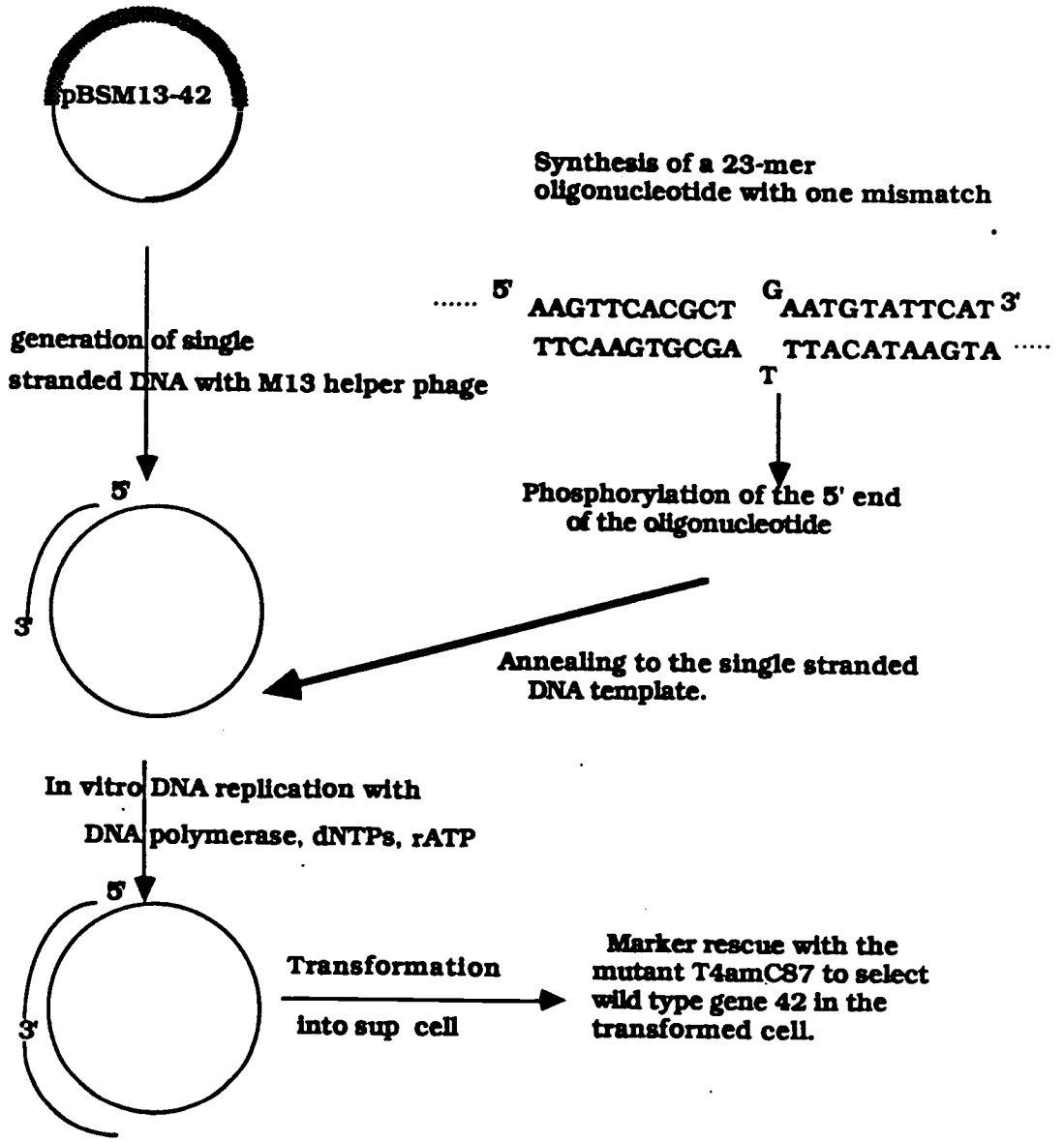


Figure II-8. A schematic representation of site directed mutagenesis, by which amber mutant T4 gene 42 was converted back to a wild type gene.

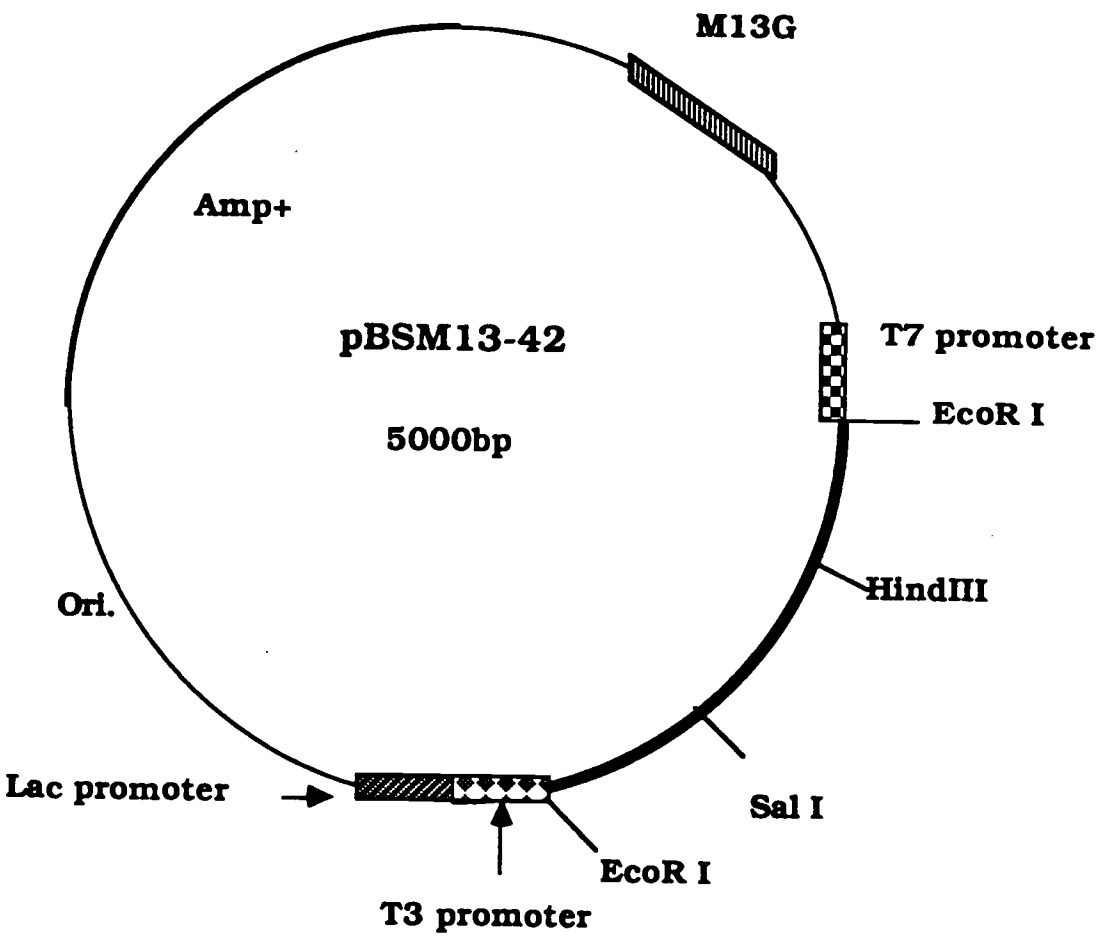


Figure II-9. Restriction map of pBSM13-42, constructed by inserting T4 gene 42 into EcoRI site of pBSM13.

pBSM13-42 were grown and superinfected with helper phages (VCS), and single-stranded DNA was isolated from the generated virus after the virus were collected with polyethylene glycol. The oligonucleotide designed for the mutagenesis is a 23-mer complementary sequence to gene 42 around the amber mutation site, shown in Figure II-8. A single mismatch at the amber mutation site allows the oligonucleotide to serve both as a mutagen and as a primer to produce wild-type gene 42 in a DNA polymerization and ligation reaction. At the end of the reaction, one half of the population of the DNA molecules still contain mutant gene 42 while the other half of the population should contain wild-type gene 42. After the *in vitro* DNA replication reaction, the reaction mixture was transformed into a suppressor minus *E. coli* cell, and the transformants were selected by their resistance to ampicillin. To select the clones carrying wild-type gene 42, marker rescue was done with a gene 42 amber mutant, C87, bearing the same mutation in gene 42 as the one been cloned. Marker rescue positive transformants were further confirmed by hydroxymethylase enzyme assay as shown in Figure II-10. The recombinant plasmid carrying wild-type gene 42 was isolated and purified. The wild-type gene 42 would be subcloned into an expression vector for high production of the enzyme.

4). Overexpression of dCMP Hydroxymethylase from Wild-type Gene 42

The first expression vector system I chose to express T4 gene 42

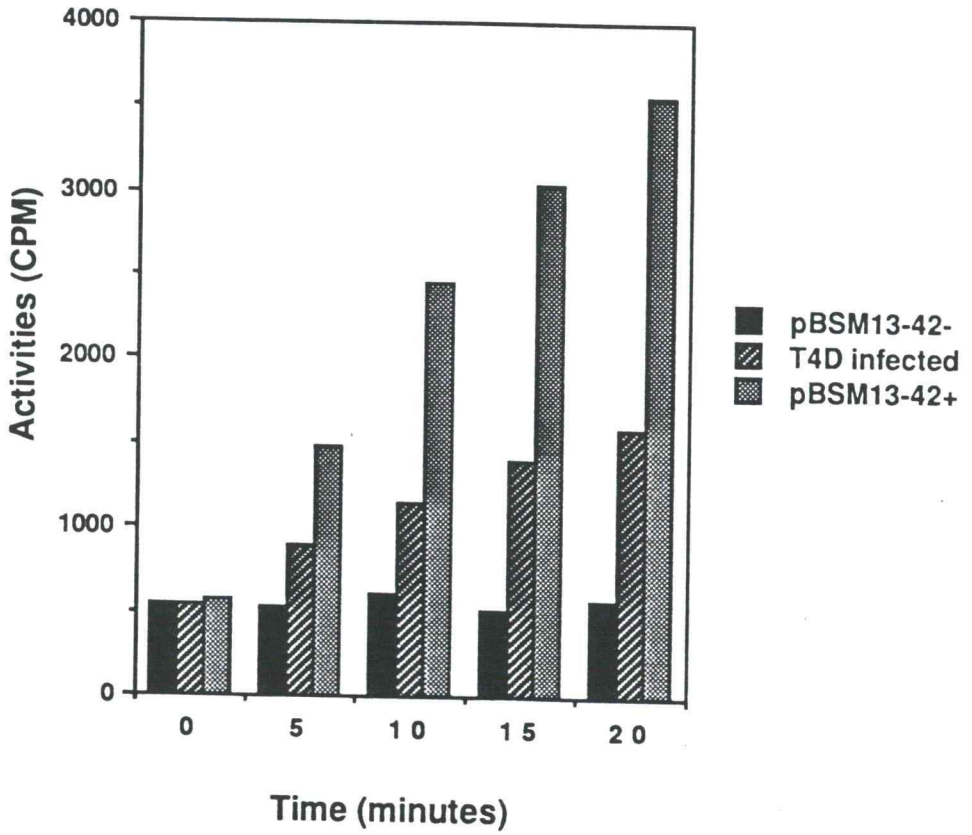


Figure II-10. Activities of dCMP hydroxymethylase expressed from cloned gene 42 in JM103 cell before and after site directed mutagenesis. Cell extract of T4D-infected *E. coli* cells was used as a control in the assays.

was a dual plasmid system. The cloning vector pPL2833 contains a thermoinducible P_L promoter followed by multiple cloning sites. The gene cloned in the multiple linker sites should be expressed through the P_L promoter under the inducing condition. The second plasmid of the system, pCI857, carries a temperature sensitive mutant gene of the repressor protein of the P_L promoter. At the permissive temperature, the repressor protein is active, and it keeps P_L promoter off so that the cloned gene cannot be expressed through the P_L promoter. At the inducing temperature, the temperature-sensitive repressor become inactive, and the cloned gene can be expressed by the released P_L promoter. This type of cloning vector with controllable promoter is very important for cloning genes whose expression is lethal to their host cells.

The wild-type gene 42 in the 1.8-kb Eco RI fragment was excised from pBSM13-42 and subcloned into the Eco RI restriction site of pPL2833 in the correct orientation. Figure II-11 shows the restriction map of the constructed plasmid and its helper plasmid, pCI857. The recombinant plasmid pPL2833-42 was transformed into $CaCl_2$-treated pCI857-containing cells. The transformant was grown at 30 C to the late exponential phase and induced at 42 C for an additional 2 hours. The cell extract of the induced cell was prepared to examine its expressed protein content by SDS gel electrophoresis and hydroxymethylase enzyme assay. Unfortunately, no protein band was shown at the position corresponding to the size of dCMP hydroxymethylase in SDS gel electrophoresis, and no elevation of enzyme activity was oberved after the cell induction. It is not clear

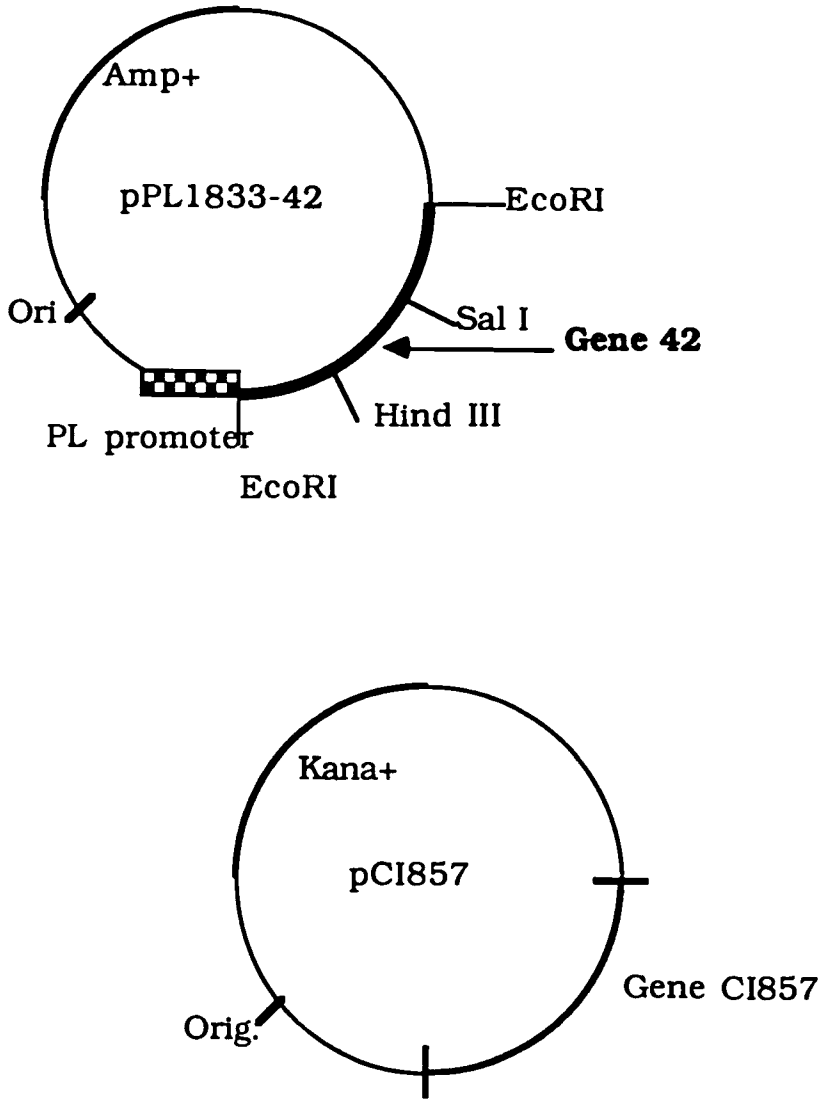


Figure II-11. Restriction maps of the constructed pPL1833-42 and its helper plasmid pCI857.

what was the cause of the failure of the expression of T4 gene 42 in the system.

The second expression system I used to overexpress T4 gene 42 was also a dual plasmid cloning system. In this system, pT7, instead of directly using P_L promoter to express cloned gene, a bacteriophage T7 promoter is used in the cloning plasmid. The second plasmid is a very large plasmid, pGP1-2, which contains a gene encoding T7 RNA polymerase under the control of P_L promoter. It also contains the gene encoding the temperature sensitive mutant form of repressor protein of P_L promoter. The restriction maps of the plasmids are shown in Figure II-12. The expression system is regulated in a cascade format. Under the noninducing condition, the active repressor protein of P_L promoter keeps the T7 RNA polymerase gene off, so that no T7 RNA polymerase is available to turn on the T7 promoter in the cloning vector. Once the system is induced, inactivation of the repressor protein releases its control on the P_L promoter. Subsequently, T7 RNA polymerase expressed through the released P_L promoter turns on the T7 promoter and expresses the cloned gene.

The wild-type T4 gene 42 was subcloned into the polylinker's Eco RI site of the cloning vector, pT7-3, in the correct orientation. The recombinant plasmids were transformed into the pGP1-2-containing *K38* cells. The transformant was grown at 30 C to the exponential phase and induced at 42 C for 30 minutes, followed by growing at 37 C for an additional 2 hours in the presence of rifampicin, which is an

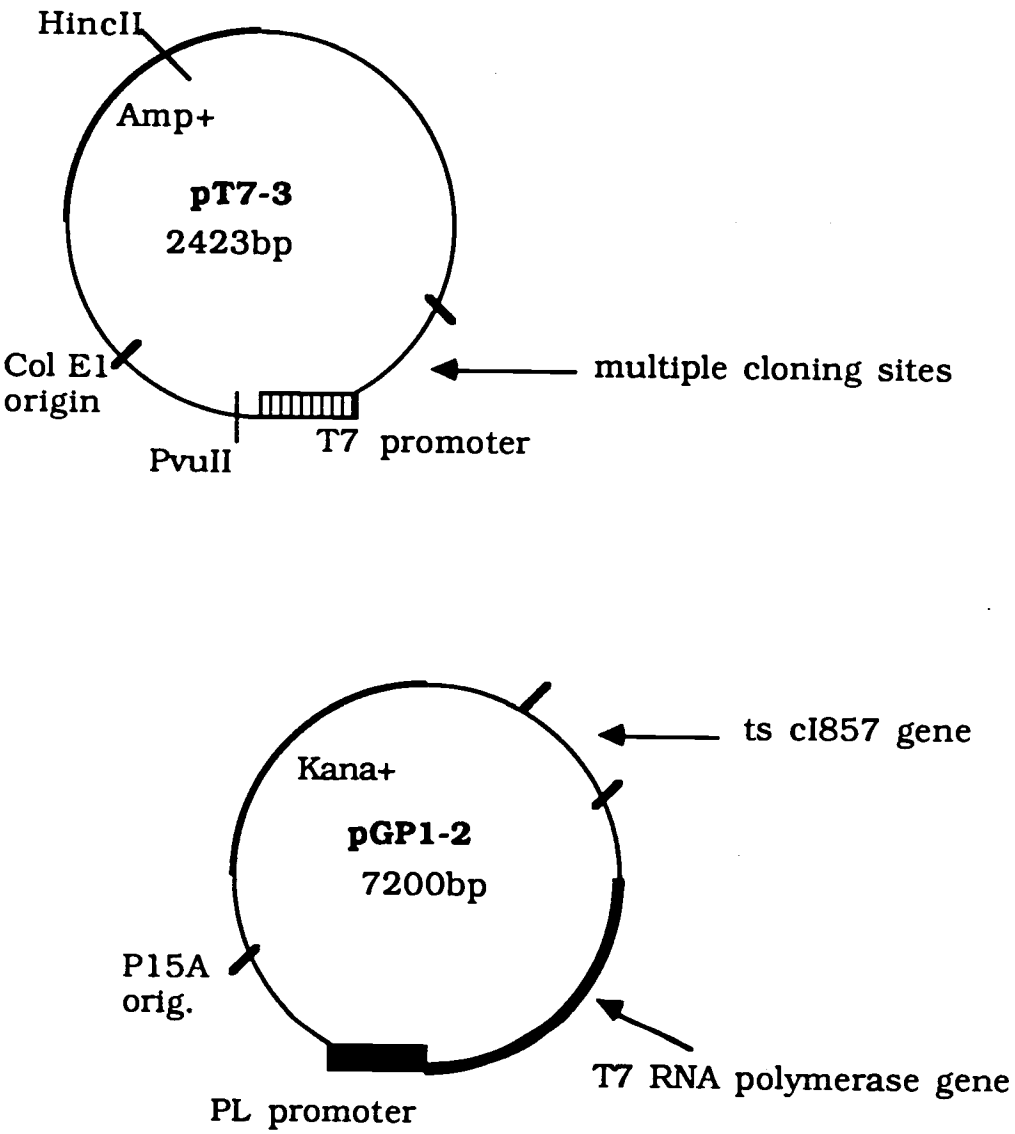


Figure II-12. Restriction maps of pT7-3 and its helper plasmid pGP1-2.

inhibitor of *E. coli* RNA polymerase. A cell extract was prepared, and the expression of the cloned gene 42 was tested by enzymatic assay and SDS gel electrophoresis. Figure II-13 shows an SDS gel of the cell extracts before and after temperature induction. A protein band with molecular weight of 28 kD was overexpressed. This is the right molecular weight for T4 hydroxymethylase. Figure II-14 shows the result of dCMP hydroxymethylase assay. The enzyme activity of the expressed cloned enzyme is fifteen times higher than the enzyme activity of a phage-infected *E. coli* cell extract.

Fifteenfold over-expression of the cloned gene 42 is not as high as we expected. With the expression system we used, the cloned gene normally can yield over 50-fold over-expression with respect to the regular level. One possible reason that the gene 42 clone is not expressed at a very high level may be interference by the flanking sequences of the gene. According to nucleotide sequence data (Lamm et al., 1988), there are about five hundred basepairs upstream of T4 gene 42 and one hundred basepairs drownstream of the gene in the 1.8-kb Eco RI DNA fragment that I cloned. Those flanking sequences, especially the upstream sequence, may inhibit the high level expression of the cloned gene.

I decided to improve the expression level of dCMP hydroxymethylase by further engineering the cloned gene 42. The strategy used to engineer the cloned gene was to trim off the flanking sequences of gene 42 with two restriction enzymes, Mst I and Sal I. Digestion of the 1.8-kb Eco RI fragment with the two restriction

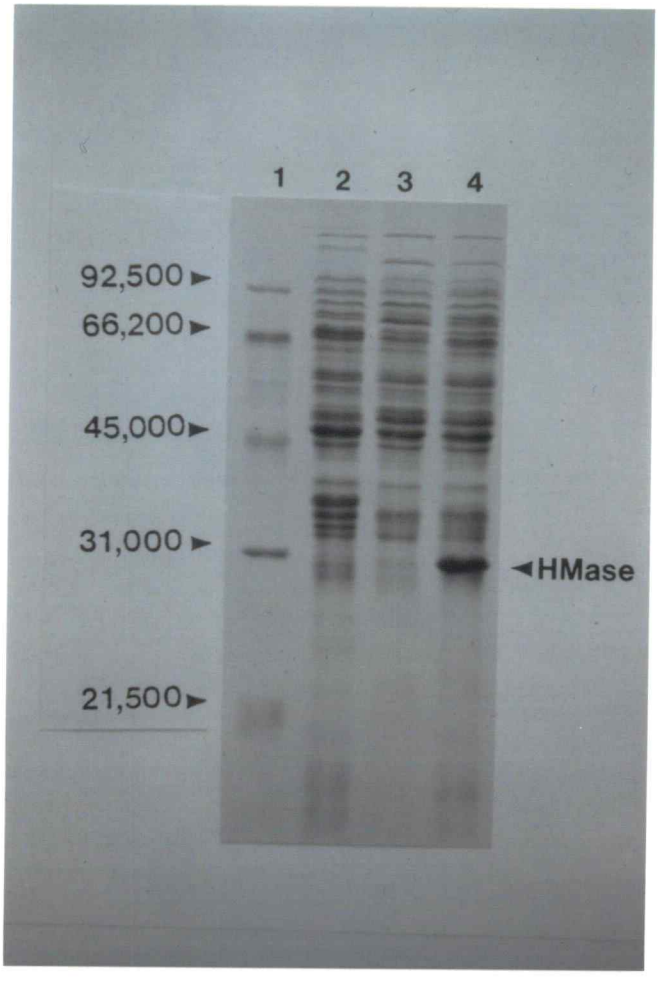


Figure II-13. Overexpressed dCMP hydroxymethylase shown on a SDS polyacrylamide gel. An arrow points to the overexpressed protein.

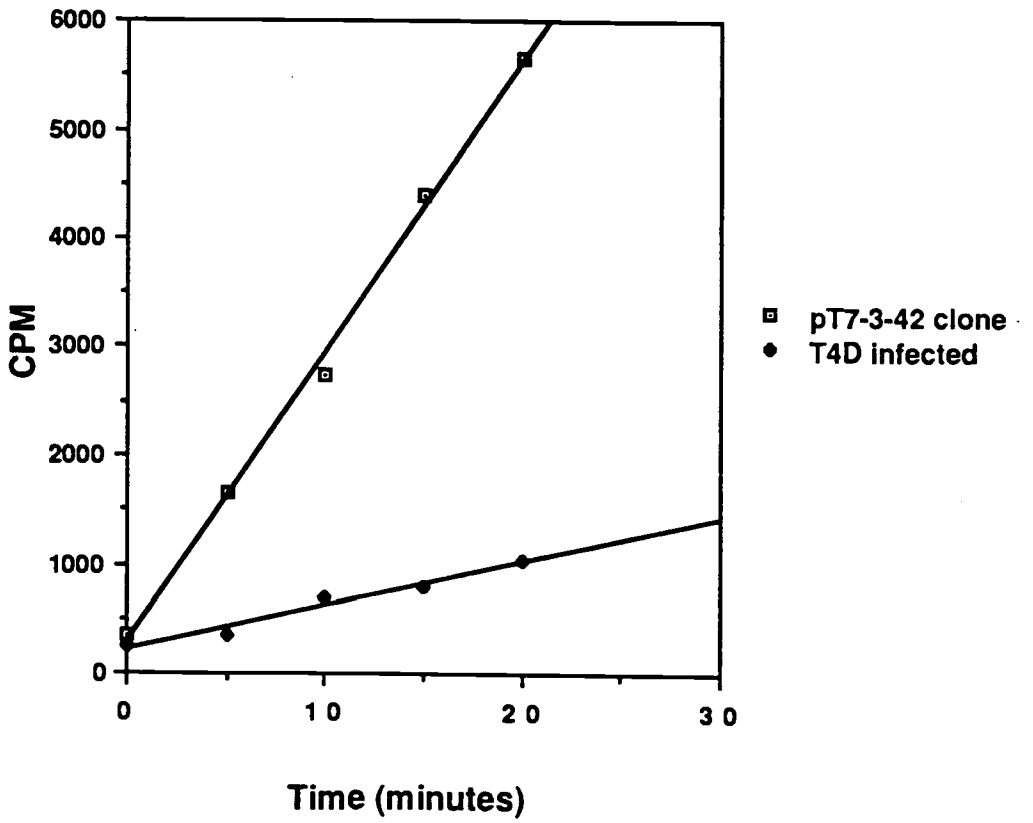


Figure II-14. Activity of dCMP hydroxymethylase expressed from pT7-42, measured by incorporation of ^{14}C into hm-dCMP in the ^{14}C assay. A T4-infected cell extract was used as a control in the assay.

enzymes would result a 1.15-kb DNA fragment which contains the whole gene 42 and very short flanking sequences. The 1.15-kb DNA fragment was inserted back to the pT7 vector, and the resulted recombinant plasmid named pT7-42 is shown in Figure II-15. Following transformation of pT7-42 into pGP1-2-containing *K38* cell, the transformant was grown, and the cloned gene was induced as described earlier. Then a cell extract was prepared and used for SDS gel electrophoresis and enzyme assay to test the level of the expressed dCMP hydroxymethylase. Both tests showed that the new gene 42 clone can over-express dCMP hydroxymethylase at a much higher level than the earlier clone, and the expression level is over fifty-fold higher than phage T4-infected *E. coli* cells.

5). Purification of dCMP Hydroxymethylase from the Cloned Gene 42-containing Cell

dCMP hydroxymethylase expressed from the cloned gene may be different from the enzyme induced in phage infection. To test the structural and enzymatic identities of the cloned gene product, I decided to determine the amino terminal sequence of the protein and also the specific activity of the purified enzyme. N-terminal amino acid sequence determination could also help us to confirm the open reading frame of gene 42 deduced from nucleotide sequence data. For the purpose described above, we needed to purify the cloned gene product. The protocol used for the purification was basically the same as that used for T6 enzyme purification by Mathews in 1962 with minor modification. The purification process includes five steps: 1,

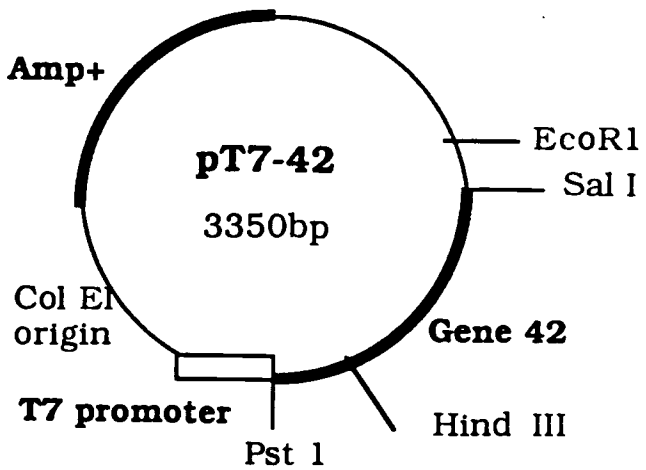


Figure II-15. Restriction map of the engineered gene 42 clone, pT7-42.

streptomycin sulfate precipitation to remove any nucleic acid from crude cell extract; 2, fractional ammonium sulfate precipitation; 3, column chromatography with DEAE cellulose; 4, chromatographic purification with hydroxylapatite column; 5, further purification with CM Sephadex column. Starting with fifteen grams of the induced gene 42-containing-cells, the purification yielded 2 milligrams of electrophoretically homogeneous dCMP hydroxymethylase. Progress of the enzyme purification is summarized in Table II-5. Figure II-16 shows an SDS gel of the protein samples through the purification. The gel shows that the protein has reached homogeneity after the purification process.

With the purified dCMP hydroxymethylase, specific activity of the enzyme was determined with [^{14}C]-formaldehyde enzyme assay. The specific activity of the purified cloned enzyme was 5865.4 nmol/min/mg calculated from the experimental result shown in Figure II-17. This value is very close to the specific activity of the enzyme pufified from T4-infected cells, 4900 nmol/min/mg. Since the molecular weight and the catalytic activity are nearly the same as the earlier reported result of the enzyme from T4-infected cells, it seems very likely that the cloned enzyme is identical to the phage enzyme. The N-terminal amino acid sequence determination of the purified hydroxymethylase was done by automated Edman degradation in an Applied Biosystems Protein Sequencer. The first 20 amino acids identified agreed precisely with the amino acid sequence deduced from the nucleotide sequence. Therefore, the open reading frame

Table II-5. Purification of dCMP hydroxymethylase

Fraction	Volume (mls)	protein concentration (mg/ml)	Specific activity (nmols/min/ml)
Cell extract	75	11.8	515.1
Streptomycin supernatant.	95	8.2	ND*
45% to 75 ammonium sulfate after dialysis.	14.7	15.2	1235
DEAE-cellulose pool after concentration and dialysis.	22	0.52	3180
Hydroxylapatite pool after concentration and dialysis	10	0.35	5250
CM-Sephadex pool after concentrated	10	0.21	5865.4

* ND= not determined

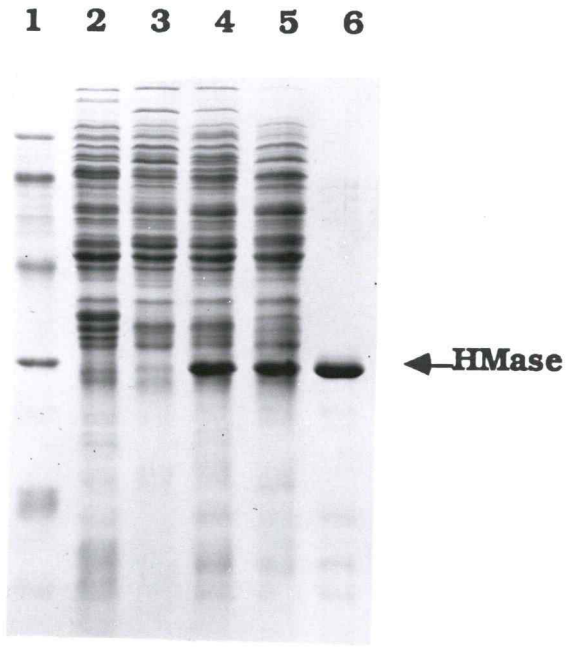


Figure II-16. SDS polyacrylamide gel showing the over-expressed dCMP hydroxymethylase from cloned gene 42, and the progress of enzyme purification. Lane 1, molecular weight marker; lane 2, T4D-infected cell extract; lane 3, cell extract of uninduced pT7-42-containing cell; lane 4, cell extract of induced pT7-42-containing cell; lane 5, induced pT7-42-containing cell extract after ammonium sulfate precipitation; and lane 6, dCMP hydroxymethylase at the end of the purification.

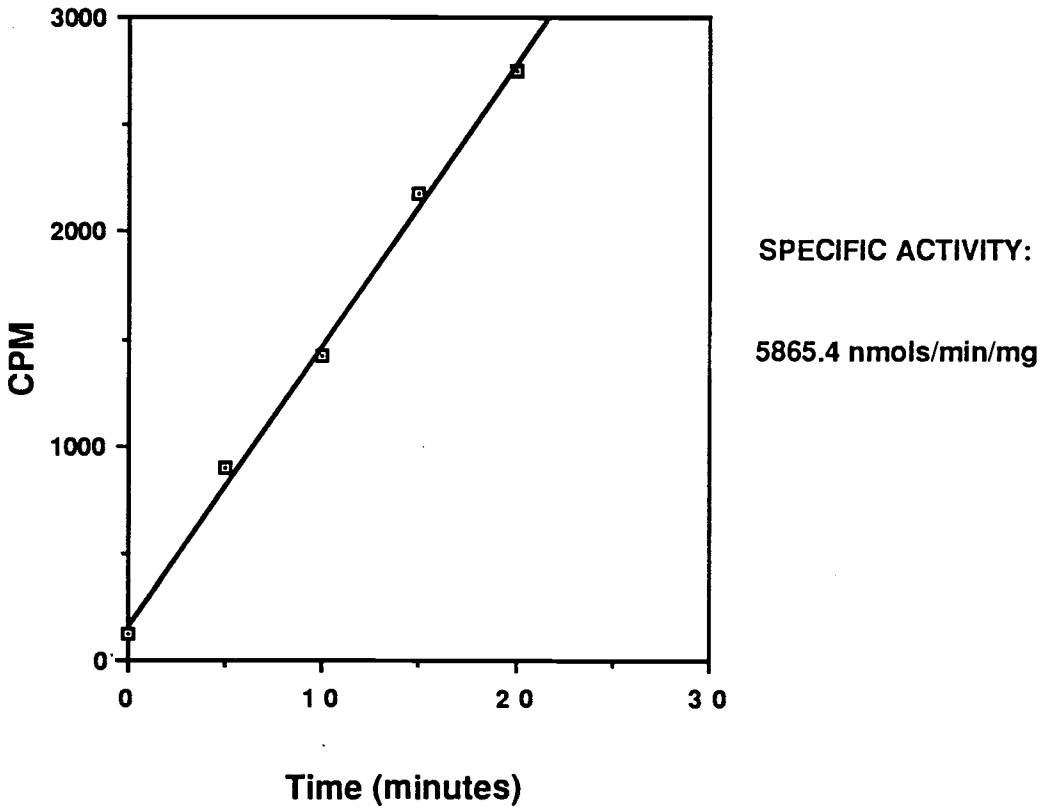


Figure II-17. Activity of purified dCMP hydroxymethylase measured by incorporation of ^{14}C into hm-dCMP as described in the Methods. The assay was done with 0.5 μg purified enzyme.

identified from the nucleotide sequence is correct.

Later on, a large scale protein purification was done, and forty milligrams of dCMP hydroxymethylase was obtained from one hundred grams of cells. The purified protein has been used in a series of studies. We have collaborated with research groups at the University of Oregon and the University of California, San Francisco, to study the structural properties of the enzyme and its enzymatic mechanism.

6). Generation of Antisera Against Purified dCMP Hydroxymethylase

With the purified dCMP hydroxymethylase, polyclonal antibody against the enzyme was generated. The antiserum was made both with column chromatography-purified protein and with protein eluted from an SDS gel. The advantage of the antiserum made with native protein is that the polyclonal antibody contains all possible epitopes against both primary structure and secondary structure. However, it is difficult to obtain a protein preparation with one hundred percent purity through column chromatography. Contamination with trace amounts of other proteins may cause high background when the antiserum is used for immune reaction assays. Elimination of the contaminants can be done by gel purification of the protein, follwed by electro-elution of the protein from an SDS gel. The gel purified protein can approach one hundred percent purity. The disadvantage of the gel purified protein is that it becomes denatured during SDS gel electrophoresis and electro-elution. As a result, the polyclonal

antiserum against the gel purified protein may not represent epitopes related to the secondary structure as well as the tertiary structure of the protein. Therefore, polyclonal antibody generated with either native or gel-purified denatured protein has both advantages and disadvantages. The choice of the antiserum to be used in a particular experiment is highly dependent upon the objective of the experiment.

Each antibody preparation involved immunization with 1 mg protein purified as described above. The quality of the each antiserum was tested by Western blotting. For Western blotting, T4-infected *E. coli* cell extract and purified dCMP hydroxymethylase were sujected to electrophoresis in an SDS polyacrylamide gel. The fractionated proteins were electro-transferred to a nitrocellulose sheet, and immune reacted with antisera against dCMP hydroxymethylase. The immune blots were developed after probing with a secondary antibody, anti-rabbit-IgG, with the results shown in Figure II-18. The immune blot with the antiserum generated with gel-purified protein has less background than the blot with antiserum generated with the native protein. Based on the blots, there are seem to be no difference between the two antisera with respect to antibody activity.

7). Optimization of Storage Conditions for the Purified dCMP Hydroxymethylase

According to previous experience in our laboratory, purified dCMP hydroxymethylase could be quite stable when stored on ice, and its enzymatic activity does not decrease much for at least four weeks.

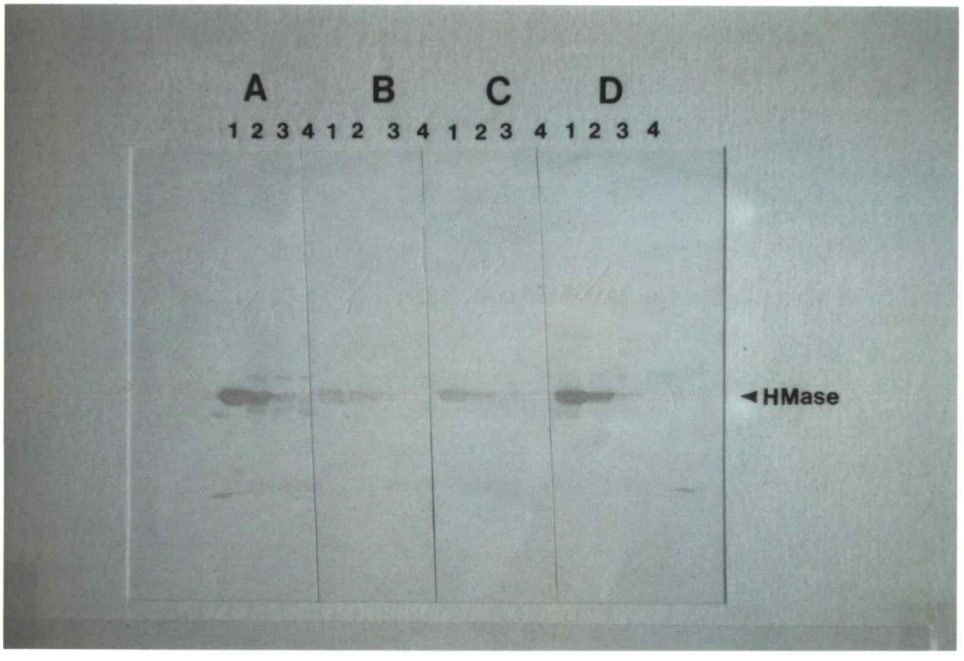


Figure II-18. Western blots showing immune reactions of dCMP hydroxymethylase with its antibody, generated either with column-purified native protein (panels A and B) or with gel-purified denatured protein (panels C and D). Protein samples used in the Western blots: lane 1, purified dCMP hydroxy-methylase; lane 2, cell extract of the induced gene 42 clone; lane 3, T4-infected cell extract; and lane 4, uninfected *E. coli* cell extract. Antisera were diluted 1000-fold (panels A and D) or 10,000-fold (panels B and C) in the reactions, respectively.

However, for long term storage of the purified enzyme, this method is inconvenient. To find a better way for storing large amounts of purified enzyme, several storage conditions were selected, and the enzyme was subjected to test those conditions. After six weeks of storage, the activities of the enzyme samples stored under those conditions were measured and compared. Table II-6 shows the storage conditions and the remaining enzyme activity. Even though the activities of the enzyme samples stored under those different conditions are not much different, it can be seen that the enzyme sample stored at -20 C with 50% glycerol maintains the highest enzyme activity. The old storage method, keeping the enzyme sample on ice, seems not a good way for long term storage of the purified enzyme based on this study.

Table II-6. Storage conditions and remaining activity of purified dCMP hydroxymethylase

Conditions	NH_4SO_4	buffer*	PMSF#	Glycerol	Temp.	Remain activity
I	/	KPO_4	1 mM	20%	-20	88%
II	/	KPO4	1 mM	50%	-20	95.5%
III	/	KPO4	1 mM	50%	-80	84%
IV	/	KPO4	/	20%	-20	87.6%
V	/	Tris-HCl	1 mM	20%	-20	78.5%
VI	25%	KPO4	1 mM	20%	-20	85.8%
VII	/	KPO4	1 mM	/	On ice	70.3%

* $KP0_4$ Buffer: 4 mM Dithiothreitol (DTT), 0.05 M KPO4, pH 6.5;

Tris-HCl buffer: 4 mM DTT, 0.05 M Tris-HCl, pH 7.5.

PMSF=Phenylmethylsulfonyl fluoride, a protease inhibitor.

4. Discussion

Bacteriophage gene 42 encoding dCMP hydroxymethylase was successfully cloned and overexpressed in *E. coli* cells. The cloning project was started with an Xho I DNA fragment from a mutant T4 phage, BK536, which synthesizes cytosine-substituted DNA. A 1.8-kb Eco RI fragment from the digestion of the XhoI fragment by Eco RI was cloned into a pUC19 plasmid vector. Genetic and biochemical examinations have indicated that the 1.8-kb Eco RI fragment contains the whole mutant gene 42, encoding dCMP hydroxymethylase. In order to make the mutant gene produce active enzyme, two approaches were attempted. The first strategy was to rescue the mutant gene in an amber suppressor cell, with reasonable amounts of dCMP hydroxymethylase being expressed from the mutant gene. Unfortunately, the effort was not successful. The mutant gene was then converted to a wild-type gene by site-directed mutagenesis. To overexpress the wild-type enzyme, wild-type gene 42 was cloned into pT7 cloning system, and the expression level of the cloned gene was over fifty times higher than that of the T4-infected *E. coli* cell. dCMP hydroxymethylase expressed from the cloned gene 42 was purified to homogeneity, and the purified enzyme was used in a series of studies. First, the N-terminal amino acid sequence of the purified dCMP hydroxymethylase was determined, and its result confirmed the open reading frame of gene 42 discovered by nucleotide sequencing (Lamm et al., 1988). Second, the specific activity of the enzyme purified from

the cloned gene was measured, and it closely agreed with the value measured with dCMP hydroxymethylase purified from phage-infected cells. Third, polyclonal antibody was generated with the purified protein. This has been used as an immunological probe to study the deoxyribonucleotide biosynthesis complex in our laboratory.

Among the four cloning vectors used in the study, two of them, pUC19 and pBSM13, have an *E. coli lac* promoter upstream of the cloning sites, and the genes cloned downstream can be selected and expressed through this promoter. When the 1.8-kb Eco RI T4 DNA fragment was cloned into the two plasmids, all of the recombinant plasmids isolated had an insertion in the same orientation; the direction of gene 42 transcription is opposite to the direction of the lac promoter. We know that *E. coli* lac promoter is a controllable promoter; it is normally turned off by its repressor protein and can be turned on by its inducer, IPTG. However, the control of the *lac* promoter by the repressor is not absolute. The genes controlled by lac promoter is constitutively expressed at a low level in the absence of a lac inducer. If the constitutively expressed gene product were lethal to its host cell, the gene could not be cloned in *E. coli* cell. In cloning the 1.8-kb Eco RI fragment downstream of the *lac* promoter, it is possible that the expression of an open reading frame from the Eco RI fragment is lethal to *E. coli*; therefore, the DNA fragment could be cloned only in one direction relative to the lac promoter. When the 1.8-kb Eco RI T4 DNA fragment containing wild-type gene 42 was cloned directly into the pT7 expression vector, the expression

level of dCMP hydroxymethylase was not as high as we expected. As mentioned earlier, the 1.8-kb Eco RI T4 fragment contains a 500-bp sequence upstream of gene 42. It is possible that this upstream sequence contains a transcription termination signal and prevents the expression of gene 42. It is also possible that the transcribed upstream sequence can form a secondary structure, which may decrease the efficiency of translation. Another possible cause of the low expression level is the direction of transcription of gene 42 related to that of the ß-lactamase. In pT7-3, the direction of transcription from the T7 promoter is opposite to the direction of ß-lactamase transcription. The transcription of ß-lactamase gene may influence the transcription of gene 42 expressed through T7 promoter from the other direction.

Availability of the cloned gene 42 and overexpressed dCMP hydroxymethylase allow us to carry out a variety of biochemical and biophysical studies of the enzyme. dCMP hydroxymethylase catalyzes hydroxymethylation of dCMP. This reaction is similar to the thymidylate synthase reaction, in that a single-carbon group is transferred to carbon-5 of a pyrimidine nucleotide, but dissimilar in that the methylenetetrafolate cofactor is not oxidized to dihydrofolate. It has been proposed that all reactions involving electrophilic substitution at carbon 5 of a pyrimidine, such as the reactions of thymidylate synthase and dCMP hydroxymethylase, proceed by a common mechanism (Santi et al., 1983). Furthermore, a comparison of the primary structure of dCMP hydroxymethylase and thymidylate synthase has revealed that a number of seemingly important amino

acid positions, including several in the active center of the enzyme, are identical (Lamm et al., 1988). Theoretically, biochemical and biophysical studies of dCMP hydroxymethylase will help to more precisely understand nucleotide and folate binding in methyl-group-transferring enzymes. Practically, since thymidylate synthase is a target enzyme in chemotherapy for inhibition of rapidly proliferating cells, understanding of reaction mechanism of dCMP hydroxymethylase will enhance the goal of developing more effective and more selective inhibitor of thymidylate synthase. We have collaborated with Daniel Santi's research group at University of California San Francisco to investigate the inhibition mechanism of dCMP hydroxymethylase by 5-fluoro-dCMP. We also have collaborated with Jim Remington's laboratory at the University of Oregon to study the crystal structure of dCMP hydroxymethylase. Recently, we started another collaboration with Larry Hardy's research group at University of Massachusetts Medical School to study the functions of some specific amino acid residues by site-directed mutagenesis.

More important, availability of large amounts of dCMP hydroxymethylase will stimulate experments directed towords its role as an element of the multienzyme complex of dNTP biosynthetic enzymes. Purified dCMP hydroxymethylase can be used to study the multienzyme complex in the *in vitro* systems, such as reconstitution of the multienzyme complex with other purified dNTP biosynthetic enzymes, cross-linking, and affinity chromatography.

III Protein-protein Interactions in Deoxyribonucleotide Synthesis: an Affinity Chromatographic Analysis of T4 Bacteriophage Deoxycytidylate Hydroxymethylase

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1. Introduction

Several aspects of procaryotic DNA replication support the notion that the DNA replication apparatus is physically coupled to a multienzyme complex of deoxyribonucleotide biosynthetic enzymes (Mathews and Sinha, 1982). First, DNA replicative chain growth is a very rapid process, about 850 nucleotides incorporated per second at 37 C, even though the affinity of the replication apparatus for its substrates is low. Second, deoxyribonucleoside triphosphates are substrates specialized for DNA replication, and are used rarely for other metabolic purposes. Numerous studies with bacteriophage T4 (Mathews and Allen, 1983; reviewed in Mathews, 1985; Moen et al., 1988) support the idea that dNTP biosynthetic enzymes are associated to form a multienzyme complex, which can efficiently synthesize and supply dNTPs to sites of DNA replication in T4-infected cells. Furthermore, association of the deoxyribonucleotide biosynthetic enzymes can effectively localize the precursors needed for dNTP biosynthesis and optimize the substrate concentrations for catalysis.

Even though the evidence obtained with cell-free enzyme aggregates (Reddy et al., 1977; Reddy and Mathews, 1978; Allen et al., 1983; and Chiu et al., 1982), as well as in vivo studies (Tomich et al., 1974; Chiu et al., 1976; Wovcha et al., 1976; Flanegan and Greenberg, 1977), support the existence of the dNTP multienzyme complex, direct physical evidence for the existence of dNTP complex or the physical associations among its protein components has not been

easily obtained (Chiu et al., 1982; Allen et al., 1983). In order to obtain definitive information about the physical existence of the dNTP synthesis complex in procaryotic system, studies with in vitro system, such as chromatographic purification, have been carried out and yielded some valuable information (Moen et al., 1988). To further understand the protein-protein interactions involved, some alternative approaches, including affinity chromatography and cross-linking, are potentially informative.

Affinity chromatography is a very useful tool in biochemical studies. For analytic studies, affinity chromatography has been used extensively to study protein-protein interactions. Formosa et al. (1983) successfully studied the interaction of T4 gp32, single stranded DNA binding protein, with other T4 DNA replication proteins by affinity chromatography technique. In their study, gp32 was coupled to Affi-Gel(Bio-Rad) to make an affinity column. Several DNA replication proteins were found to specifically interact with gp32 immobilized on the column. These include the products of gene 45 (DNA polymerase accessory protein), gene 43 (T4 DNA polymerase), gene 32, and gene uvs X. The identities of the proteins were determined by autoradiography following SDS gel electrophoresis or two-dimensional gel electrophoresis.

To study protein-protein interactions of the deoxyribonucleotide biosynthetic enzymes, we have adapted the affinity chromatography system for our study. dCMP hydroxymethylase, an essential enzyme for T4 DNA precursor biosynthesis, was immobilized on an Affi-Gel.

The generated affinity column was used to study the proteins that can specifically interact with dCMP hydroxymethylase.

2. Materials and Methods

1). Materials

Escherichia coli strain B (wild-type) and wild-type phage strain T4D have been maintained in this laboratory for some time. dCMP hydroxymethylase was purified from *E. coli* K38 containing cloned T4 gene 42 (Lamm et al., 1988). ^{35}S-methionine used for labeling proteins was purchased from New England Nuclear, and Affi-Gel 10 used for preparation of the affinity columns was purchased from Bio-Rad.

2). Coupling of Purified dCMP Hydroxymethylase to Affi-Gel

Affi-Gel 10 offers rapid, high efficiency coupling for all ligands that have a primary amino group. It is an N-hydroxysuccinimide ester of a derivative crosslinked agarose gel bead support, and it contains a neutral 10-atom spacer arm. 2.5 mg of purified dCMP hydroxymethylase was dialyzed in 0.1 M MOPS buffer, pH 7.5, and its enzyme activity was assayed before coupling to the Affi-Gel. To prepare a 2-ml column, approximately 1 ml of activated matrix was washed into coupling buffer (0.1 M MOPS, pH 7.5) at 0 C according to the manufacturer's instructions. Coupling reaction was carried out with the washed Affi-Gel and 2.5 mg of dCMP hydroxymethylase overnight at 4 C, with shaking at 4 C for at least 12 hours. Any remaining active groups were blocked by adding 0.1 ml of 1M ethanolamine-HCl, pH 8.0, per ml of gel, and agitating for another 2

hours. The coupled matrix was used to pack an affinity column in a 3-ml syringe, and the uncoupled protein was washed off extensively with a high salt washing buffer. The washing buffer is composed of column buffer (0.02 M Tris-HCl, pH 8, 1 mM EDTA, 1 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 0.05 M NaCl, 0.5 mM PMSF, 10% (v/v) glycerol) and additional NaCl to 4.0 M. The washed column was then equilibrated with the column buffer and stored at 4 C. In addition, a bovine serum albumin (BSA) control column was prepared by substituting BSA for the dCMP hydroxymethylase. The coupling conditions for BSA column were identical to those for the hydroxymethylase column, except that an additional 80 mM $CaCl_2$ was present. The coupling efficiencies were determined by measuring the protein concentrations of the reaction supernatant and wash fractions, either through Coomassie Blue Dye Protein Assay or SDS gel electrophoresis. Both of these methods indicated that more than 85% of the applied proteins became covalently bound to the matrix. For longer periods of storage, the protein-Affi-Gel columns were washed with column buffer containing 50% (vol/vol) glycerol and stored at -20 C.

3). Radioactive Labeling of T4-infected and Uninfected *E. coli* Cells, and Preparation of Cell Extracts

A 500-ml culture of *E. coli* B was grown to a density of 5×10^8 cells per ml at 37 C in M9 minimal medium and infected with T4 phage at a multiplicity of 10. Before infecting the cell culture, L-tryptophan was added to a final concentration of 25 μg/ml. To label

phage-induced proteins, equal aliquots of [^{35}S] methionine (0.5 mCi total) were added at 0, 5 and 10 minutes after infection. At 12 minutes after infection, unlabeled methionine was added to a concentration of 20 μg/ml and the culture was chilled in an ice bath. The cells were harvested by centrifugation at 5,000 x g for 20 minutes, the supernatant was discarded, and the pellet was stored at -80 C.

Cell extracts were prepared by suspending the frozen pellet in 10 ml of the column buffer and sonicating four times with 60-second blasts with a Branson sonifier at maximum power; the temperature was maintained near 4 C. Cell debris was removed by centrifugation at 10,000 x g for 15 minutes, at 4 C, and the supernatant was ready for applying to the affinity column. Incorporation of the radioactive label was measured with TCA-precipitated cell extract, and it showed that 50% of the [^{35}S] methionine label was incorporated into protein.

In addition, an 500-ml cell culture of uninfected *E. coli* B was grown to a density of 5 x 10^8 cells per ml at 37 C in M9 minimal medium and labeled by a single addition of 0.5 mCi [^{35}S] methionine. Labeling was allowed to proceed for 12 minutes, and then the cells were harvested. A cell extract of uninfected *E. coli* was prepared in the same way as described above.

4). Western Blotting

Equal volumes of the protein samples eluted from the affinity columns were concentrated by TCA precipitation and electrophoretically fractionated in an 10% SDS/polyacrylamide gel and

electro-transferred to a nitrocellulose membrane. 1:500 dilutions of primary antisera and 1:1000 dilutions of secondary antibody (phosphatase-conjugated anti-rabbit IgG, Sigma) were used in immune reactions as described previously (Blake et al., 1984).

5). Two-dimensional Gel Electrophoresis

a. Preparation of protein samples for two-dimensional gel electrophoresis (Kutter, personal communication)

To prepare a radioactive labeled T4-induced protein sample, A 5-ml culture of *E. coli* B was grown, infected, and ^{35}S-methionine labeled, as described above. The culture was chilled briefly on ice and then was centrifuged for 10 minutes at 12,000 g. The cell pellet was resuspended in 250 µl of SDS lysis buffer (0.0625 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, and 2% SDS). The suspension was transferred to a 1.5-ml Eppendorf tube and was sonicated for 1.5 minutes with three 30-s blasts interspersed with 1 minute of chilling on ice. The sonicated cell suspension was centrifuged for 15 minutes in a microfuge at 4 C to remove unbroken cells and debris. To each 100 µl of supernatant solution, 100 mg of urea was added and dissolved by warming very briefly at 37 C. Then 200µl of NEPHGE buffer was added to each 100 µl solution, and the sample was quick-frozen and stored at -80 C.

To prepare column-eluted protein sample for two-dimensional gel electrophoresis, protein was concentrated by TCA precipitation, and the pellet was resuspended in NEPHGE sample buffer. The sample

was stored at -80.

b. First dimensional gel electrophoresis

Nonequilibrium pH gradient gels were made in glass tubing of 130 x 2.5 mm inside diameter. as described (O'Farrell et al., 1977). The tubing gels contained a final concentration of 1% ampholines at pH 5 to 7 and 1% ampholines at pH 3.5 to 10. The radioactive labeled samples prepared as described above, and containing 5 X 10^5 acid-precipitable cpm in a volume of 10 to 50 µl, were loaded in the tubing gels. The gels were run at 400 volts for 4 hours with the cathode on the bottom and anode on the top, at constant voltage. At the end of the run, the gels were removed by careful extrusion and equilibrated for 1 hour in SDS sample buffer (0.005% Bromphenol blue, 0.0625 M Tris-HCl pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, and 2% SDS), and were stored at -70 C.

c. Second dimensional gel electrophoresis

An SDS polyacrylamide gel of 1.5 mm thickness with a 2.5-cm 4% slacking gel and a 12-cm 12.5% separating gel was used. The first dimension cylindrical gel was thawed and laid on the stacking gel. The gels were subjected to electrophoresis for approximately 5 hours at 30 milliamperes of constant current for each gel until the marker of Bromphenol blue dye reached the bottom of the gels. For fluorography, the gels were treated with DMSO and PPO, and were dried on Whatman 3 MM paper and exposed to X-ray film.

3. Results

1). Affinity Chromatography of T4-encoded Proteins with dCMP Hydroxymethylase Affinity Column

To study protein-protein interactions of deoxynucleotide biosynthetic enzymes, we attempted to adapt the affinity chromatographic system for our study. Our strategy was to make an affinity column with purified dCMP hydroxymethylase expressed from its cloned gene and the activated affinity gel support, Affi-Gel 10. Two and one half milligram of the purified dCMP hydroxymethylase was immobilized on Affi-Gel, and a 2.0-ml affinity column was prepared. Bovine serum albumin was used to prepare a control column for the study. *E. coli* cells were infected by T4 bacteriophage and the bacteriophage-encoded proteins were labeled with ^{35}S-methionine. One portion of the radioactive labeled proteins was applied to the dCMP hydroxymethylase affinity column, and an identical portion was applied to the bovine serum albumin column. Each column was washed extensively with column buffer containing 50 mM NaCl, and then the specifically bound proteins were eluted from the column with column buffer containing 500 mM NaCl. Much more radioactive protein was retained by the dCMP hydroxymethylase column than by the albumin column, as shown in Figure III-1. In addition, it was interesting to know wether any *E. coli* proteins could also specifically bind to the dCMP hydroxymethylase column. A radioactive labeled cell extract prepared from uninfected *E. coli* cells was

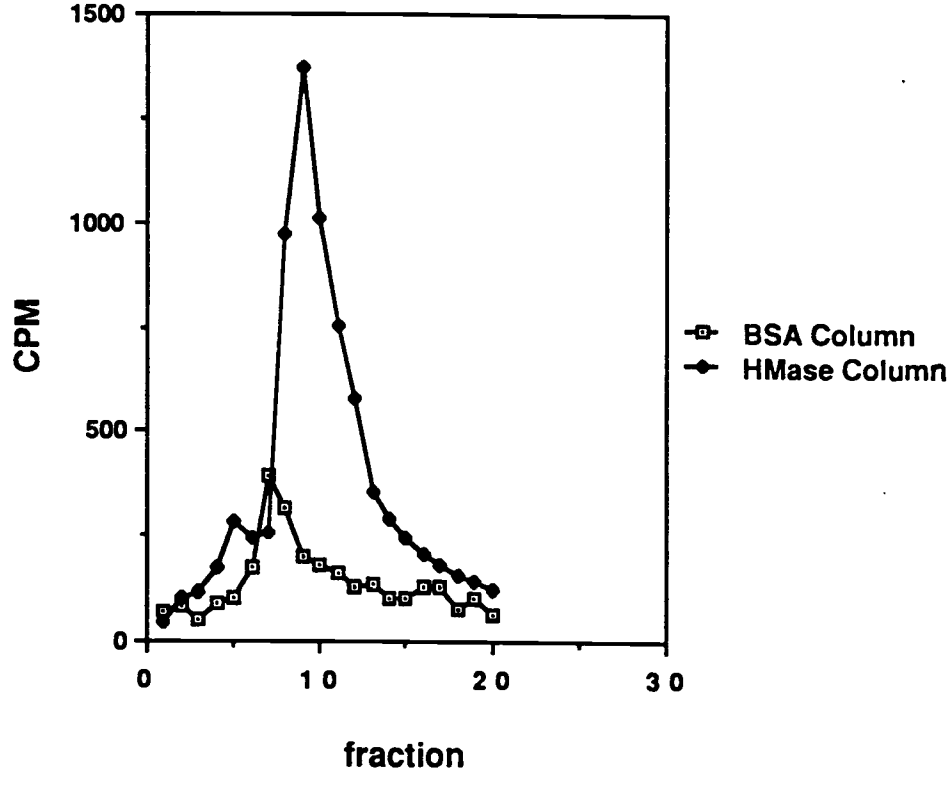

Figure III-1. Elution profiles of T4-induced proteins retained by the dCMP hydroxymethylase affinity column and the bovine serum albumin control column. The size of each fraction was 0.5 ml. Radioactivity of 5 μl of each fraction was measured and plotted.

chromatographed with the dCMP hydroxymethylase column and the albumin column. In Figure III-2, it can be seen that little more radioactive *E. coli* protein was retained by the dCMP hydroxymethylase column than by the albumin columns.

2). Analysis of the Affinity Column-Retained Proteins with SDS Gel Electrophoresis Followed by Fluorography

In order to identify the specific proteins binding to the columns in the experiments shown in Figure III-1 and Figure III-2, the protein fractions with high radioactivity were pooled, concentrated by TCA precipitation, and were fractionated by SDS/polyacrylamide gel electrophoresis. The radioactive proteins were detected by fluorography, as shown in Figure III-3. It can be seen that no T4-encoded protein binds specifically to the albumin column; likewise, not much *E. coli* protein binds to the dCMP hydroxymethylase column specifically. It should be noted that there is one *E. coli* protein with a molecular weight of 43-kD specifically bound to the dCMP hydroxymethylase column. In contrast, at least 9 T4 proteins bound specifically to the dCMP hydroxymethylase column. Because the molecular weights of most of the deoxyribonucleotide biosynthesis enzymes are known, shown in Table I-1, the identity of some of the proteins can be predicted by this one-dimensional gel electrophoretic analysis. As shown in lane 5 of Figure III-3, the molecular weights of several protein species retained by the dCMP hydroxymethylase column match the molecular weights of the following dNTP biosynthesis enzymes: dCMP hydroxymethylase (a homodimer, subunit

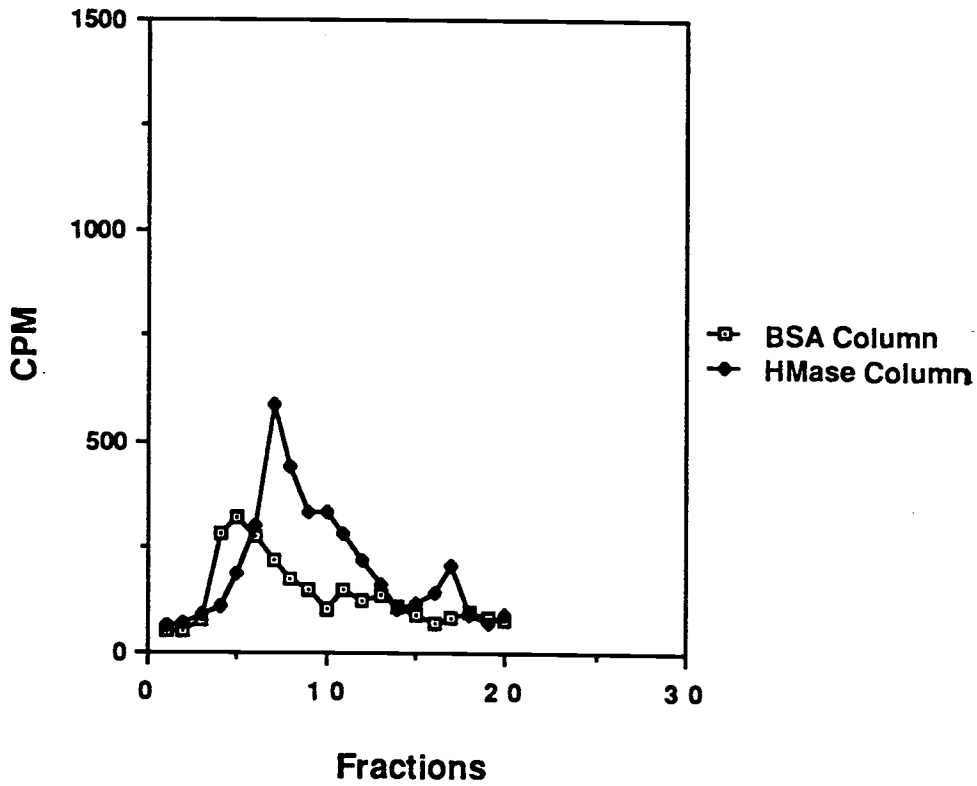

Figure III-2. Elution profiles of *E. coli* proteins retained by the dCMP hydroxymethylase affinity column and the bovine serum albumin control column. The fraction size was 0.5 ml. Radioactivity of 5 µl of each fraction was measured and plotted.

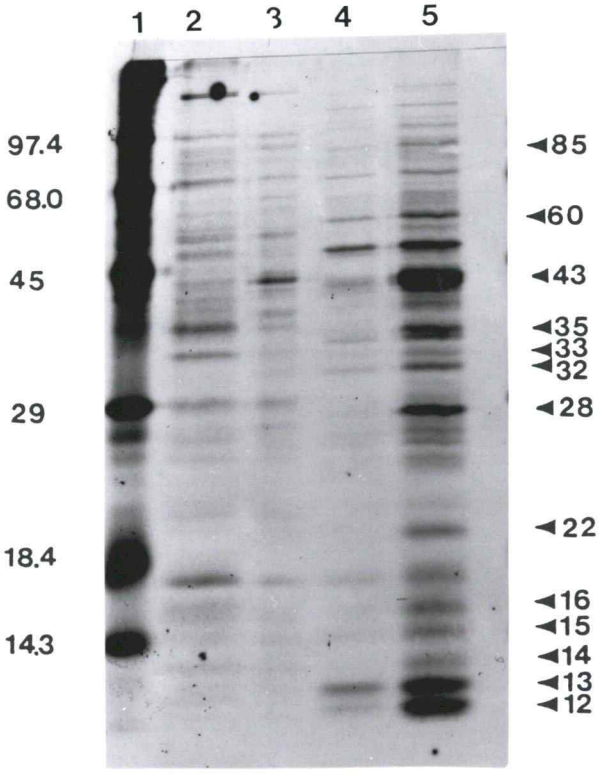

Figure III-3. Autoradiogram of an SDS polyacrylamide gel showing the proteins from dCMP hydroxymethylase (HMase) affinity chromatography. lane 1, protein molecular weight marker; lane 2, *E. coli* proteins eluted from bovine serum albumin (BSA) column; lane 3, *E. coli* proteins eluted from HMase column; lane 4, T4-induced proteins eluted from BSA column; and lane 5, T4 induced proteins eluted from HMase column.

Mr=28 kD), thymidylate synthase (a homodimer, subunit Mr=33), dNMP kinase (subunit Mr=22), small subunit of ribonucleotide reductase (subunit Mr=36), and NDP kinase (subunit Mr=15.5).

3). Identification of the Proteins Specifically Bound to dCMP hydroxymethylase Affinity Column by Western Blotting

To further identify T4 protein proteins specifically bound to the dCMP hydroxymethylase, a Western blotting experiment was performed. Antisera used for the immune reaction assay include anti-T4 thymidylate synthase, anti-T4 dihydrofolate reductase, anti-T4 ribonucleotide reductase, anti-T2 dCMP deaminase, anti-T4 thioredoxin, and anti-T4 dCMP hydroxymethylase. T4-induced proteins retained by dCMP hydroxymethylase column were separated on a SDS polyacrylamide gel and electro-transferred to a nitrocellulose sheet. For the controls, an unfractionated extract of T4-infected *E. coli* cells, as well as the phage proteins eluted from the albumin column and the *E. coli* proteins eluted from the dCMP hydroxymethylase column and from the albumin column, were also separated on the same SDS polyacrylamide gel and electro-transferred to the nitrocellulose. The proteins immobilized on the nitrocellulose sheet were probed with the antisera described above and developed after immune reaction with the secondary antibody. Figure III-4 shows the result of the Western blotting. Panel 1 shows Western blots with the antiserum against dCMP hydroxymethylase. It can be seen that the antiserum specifically reacts with dCMP

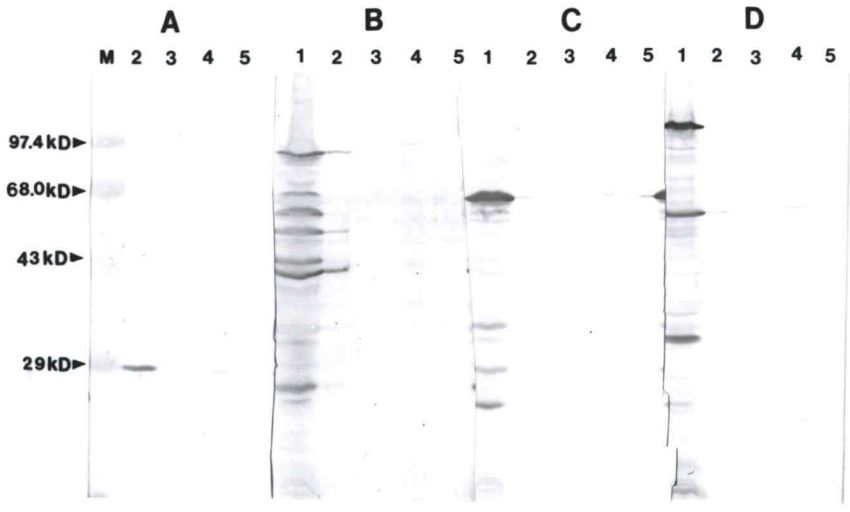

Figure III-4. Western blotting of proteins resulted from dCMP hydroxymethylase affinity chromatogrophy with antisera against T4 dNTP biosynthetic enzymes. Protein samples applied to each lane: lane 1, T4-infected cell extract; lane 2, T4 proteins eluted from dCMP hydroxymethylase (HMase) column; lane 3, E. coli proteins eluted from dCMP HMase column; lane 4, T4 proteins eluted from BSA column; and lane 5, E. coli proteins eluted from BSA column. Panel A, Western blot with anti-dCMP hydroxymethylase; panel B, Western blot with a mixture of three antisera: anti-T4 ribonucleotide reductase, anti-T4 thioredoxin, and anti-T2 dCMP deaminase; panel C, Western blot with anti-T4 dihydrofolate reductase; and panel D, Western blot with anti-T4 thymidylate synthase.

hydroxymethylase in the phage-infected protein sample eluted from dCMP hydroxymethylase column in lane 1 but not in the control lanes. The existence of dCMP hydroxymethylase in the specific eluted protein sample indicates that the immobilized dCMP hydroxymethylase can interact with at least one of the dCMP hydroxymethylase subunits labeled in phage-infected cells. The second panel of the figure shows the immune reaction with a mixture of three antisera: anti-T4 ribonucleotide reductase, anti-T4 thioredoxin, and anti-T2 dCMP deaminase. The three antisera can immune react with several T4 induced proteins in phage-infected cell extract, as shown in lane 1, and they also react with some phage proteins retained by dCMP hydroxymethylase affinity column showed in lane 2. Based on the molecular weights of the immune reacted protein bands, two of them have the same molecular weights as the two subunits of T4 ribonucleotide reductase. Therefore, phage-encoded ribonucleotide reductase apparently can interact with dCMP hydroxymethylase immobilized on the affinity column. Again, the antisera do not immune-react with any proteins in the control lanes. Panel 3 shows the immune reaction with the antiserum against T4 dihydrofolate reductase, while panel 4 shows the immune reaction with the antiserum against T4 thymidylate synthase. Lane 1 in each panel shows the immune-reacted T4 induced proteins of the phage-infected *E. coli* cell extract. None of the proteins eluted specifically from the dCMP hydroxymethylase affinity column can show immune reaction with the two antisera. This means either that the column-

eluted proteins do not contain T4 dihydrofolate reductase and thymidylate synthase at detectable levels, or the two enzymes do not interact with the immobilized dCMP hydroxymethylase at all.

4). Two-Dimensional Gel Electrophoresis to Further Identify the Proteins Resulting from Affinity Chromatography

A more precise way to identify proteins that bound to the dCMP hydroxymethylase column is two-dimensional gel electrophoresis (O'Farrell et al., 1977). Bacteriophage T4-encoded proteins have been identified either by analysis of various T4 mutants, or with purified phage proteins by two-dimensional gel electrophoresis (Cook and Seascholtz, 1982; Burke et al., 1983; Cowan et al., 1989, unpublished report). By using previous information about the two-dimensional gel pattern of T4 proteins as reference, we attempted to identify the proteins that bound to the dCMP hydroxymethylase column. The column-eluted proteins were separated by nonequilibrium pH gradient electrophoresis in the first dimension, then were further resolved by SDS polyacrylamide gel electrophoresis followed by fluorography. To generate a standard T4 protein pattern for identifying the T4 proteins retained by the dCMP hydroxymethylase column, unfractionated radioactive labeled T4-infected *E. coli* cell extract and uninfected cell extract were also subjected to two-dimensional gel electrophoresis. Figure III-5 and Figure III-6 show two-dimensional gel patterns of T4-infected and uninfected cell extracts. By comparing the protein pattern of T4-infected cell extract with that of uninfected cell extract, T4 proteins could be distinguished from *E. coli* proteins.

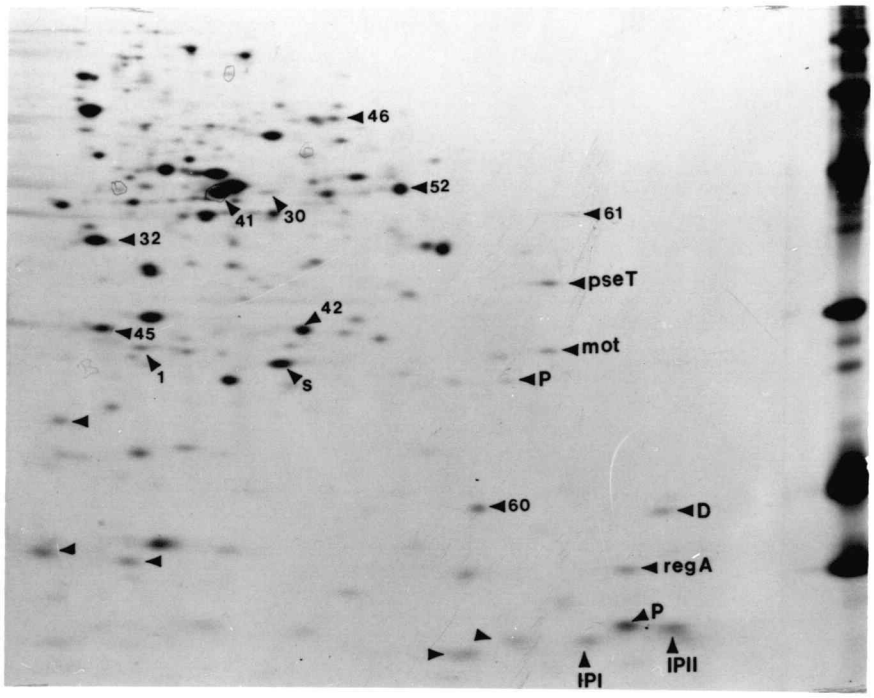

Figure III-5. T4-encoded proteins electrophoretically separated on a two dimensional gel followed by fluorography. The arrows piont to the T4-induced gene products.

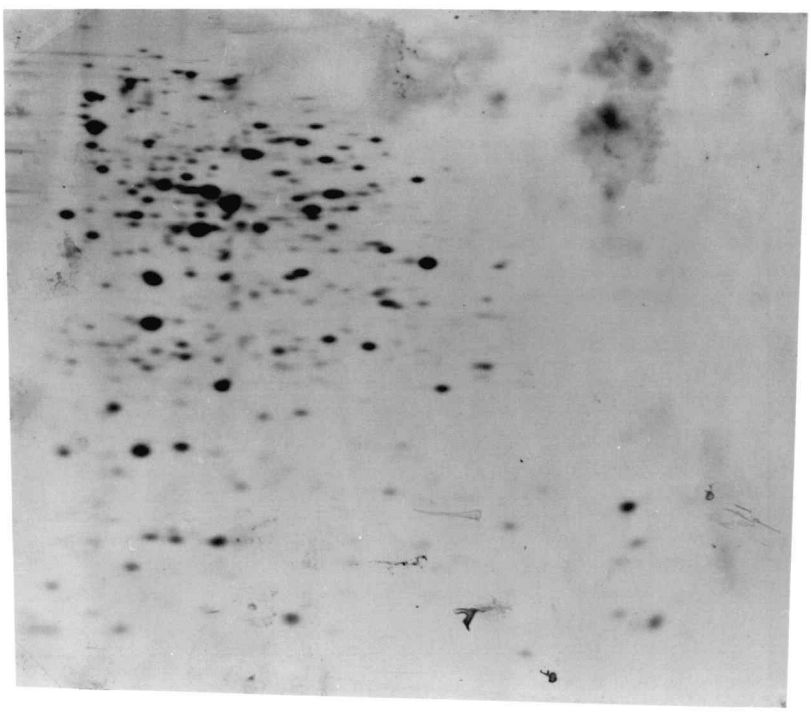

Figure III-6. *E. coli* proteins electrophoretically separated on a two dimensional gel followed by fluorography.

Phage-encoded proteins were then identified with references to previous studies described above. Figure III-7 shows the dCMP hydroxymethylase column-retained proteins that were separated by two-dimensional gel electrophoresis. Based upon the positions of the proteins on the two-dimensional gel, some of the proteins were identified as shown in Figure III-7. Surprisingly, several DNA replicative proteins were retained by the dCMP hydroxymethylase affinity column, in addition to two dNTP biosynthetic enzymes, ribonucleotide reductase and dCMP hydroxymethylase.

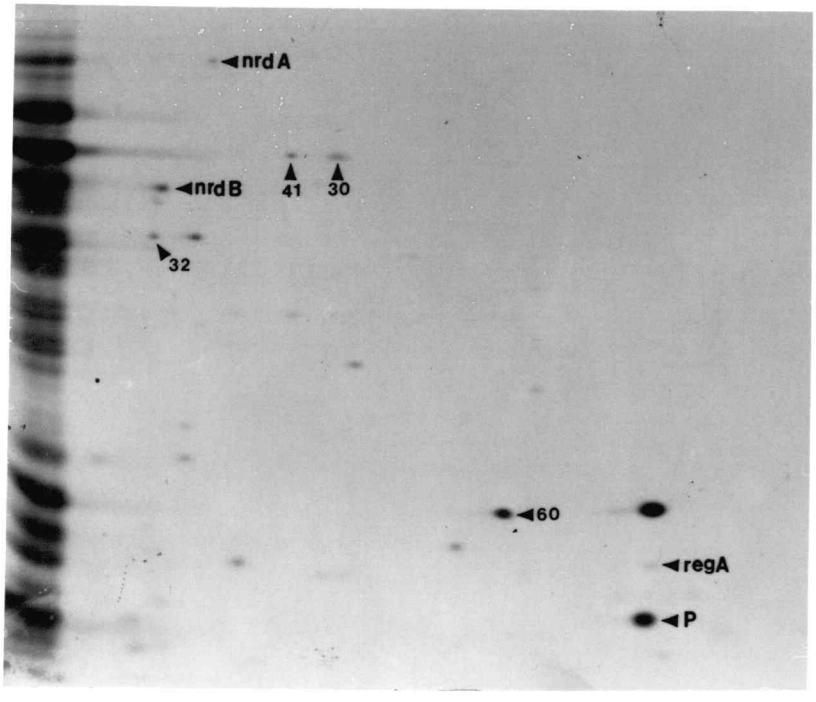

Figure III-7. Analysis of the proteins eluted from the dCMP hydroxymethylase affinity column by two dimensional gel electrophoresis. The arrows point to the column retained T4 gene products.

4. Discussion

In the present study, we have investigated protein-protein interactions among deoxyribonucleotide biosynthetic enzymes of bacteriophage T4 by affinity chromatography. T4-encoded dCMP hydroxymethylase was coupled to an activated agarose gel supporter, Affi-Gel, and the affinity column was used to chromatograph radioactive labeled T4-infected cell extract and uninfected *E. coli* cell extract. The proteins retained by the dCMP hydroxymethylase affinity column were analyzed by SDS gel electrophoresis, Western blotting, and two-dimensional gel electrophoresis. Several affinity column-retained proteins have been identified by those methods.

The identified T4 proteins, retained by the dCMP hydroxymethylase affinity column, include ribonucleotide reductase, RegA (a translational repressor protein), and several DNA replicative proteins. Earlier study from this laboratory has revealed that both T4-encoded ribonucleotide reductase and RegA are essential for assembly of the dNTP biosynthesis complex (Moen et al., 1988). The conclusion was based on the fact that infection of T4 strains bearing mutation either in RegA or one of the nrd genes does not form dNTP biosynthesis complex in *E. coli* cells. Since our result presented here shows that both ribonucleotide reductase and regA can specifically interact with dCMP hydroxymethylase, it is possible that those enzymes physically associate to one another in T4 dNTP biosynthesis complex.

It has been proposed that the dNTP biosynthesis complex is physically coupled to DNA replication apparatus for efficiently supplying dNTPs during DNA replication in procaryotic organisms (Mathews and Sinha, 1982). However, there has been no direct evidence supporting the hypothesis. Interaction of dCMP hydroxymethylase with DNA replicative proteins shown in this study is the first evidence supporting the physical coupling of the two DNA metabolic processes.

Our result shows that only one dNTP biosynthetic enzyme, ribonucleotide reductase, was retained by the dCMP hydroxymethylase affinity column at the detectable level. It is possible that the chromatographic condition used in our study is not optimal for the interaction of dCMP hydroxymethylase with other dNTP biosynthetic enzymes. For instance, substrates or cofactors of the enzymes may be necessary for such interactiones. Alternatively, dCMP hydroxymethylase may not directly contact other proteins in the complex. Such interactions can be explored by immobilizing other purified dNTP biosynthetic enzymes and identifying proteins that bind to these proteins.

IV Analysis of T4 Phage Deletion Mutant Lacking *td* and *frd* Genes

Running title: T4 *td* and *frd* Deletions

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1. Introduction

The genome of bacteriophage T4 encodes most of its own enzymes for deoxyribonucleotide biosynthesis and DNA replication. Some of the enzymes duplicate and augment the functions of host cell enzymes, helping thereby to support the high rate of DNA synthesis in T4 phage-infected cells. Thymidylate synthase and dihydrofolate reductase, which are involved in thymine nucleotide biosynthesis, are two such enzymes. In addition to their catalytic functions, the two enzymes are also structural components of the phage tail baseplate (Capco and Mathews, 1973; Kozloff et al., 1970; Mathews, 1971). The structural role played by these proteins is supported mainly by three lines of evidence: (a) detection of low levels of enzyme activities in purified viral particles (Kozloff et al., 1975; Kozloff et al., 1977; Kozloff et al., 1970; Mosher et al., 1977); (b) neutralization of T4 phage infectivity by antiserum against either of the enzymes (Capco and Mathews, 1973; Mathews et al., 1973); (c) the observation that genes coding for the two enzymes are determinants of the heat lability of the virion (Capco and Mathews, 1973; Kozloff et al., 1975; Kozloff et al., 1975; kozloff et al., 1977; Mathews, 1971).

Some years ago, we studied three deletion mutants that had been characterized by Homyk and Weil (1974) -- *del*(63-32)1, *del*(62-32)7, and *del*(63-32)9 -- hereafter called *del*1, *del*7, and *del*9, respectively. According to DNA heteroduplex mapping, two of the mutants, *del*7 and *del*9, are deleted for both the *td* gene, encoding

thymidylate synthase, and the *frd* gene, encoding dihydrofolate reductase. The deletion mutants were used in attempts to confirm that thymidylate synthase and dihydrofolate reductase are T4 baseplate components. When neutralization of infectivity was studied, unexpectedly, the mutants were found to be even more readily neutralized than wild-type T4D by antisera against either T4 thymidylate synthase or dihydrofolate reductase (Capco and Mathews, 1973; Mosher et al., 1977). Moreover, deletion mutant-infected *E. coli* cell extracts contain proteins which can cross-react with the antisera, as shown by immunodiffusion experiments (Capco and Mathews, 1973; Mosher et al., 1977). These results are unexpected if the proteins are completely missing from the virion. One possible explanation for these paradoxical results was that the *td* gene and *frd* gene are not completely deleted in the mutants, and the remaining parts of these genes can still produce cross-reacting materials to interact with thymidylate synthase and dihydrofolate reductase antibodies (Mosher and Mathews, 1979).

To understand the apparent paradox and to elucidate the structural roles of thymidylate synthase and dihydrofolate reductase, it is important to carefully define the deletion mutants. In this investigation, we characterized the mutants with Southern blotting analysis, along with immunological and enzyme assays. We also made new antisera to T4 thymidylate synthase and dihydrofolate reductase purified from *E. coli* cells containing either the cloned T4 *td* gene or the *frd* gene. With the precisely characterized T4 deletion mutants

and newly made antibodies, we re-examined the experiments supporting the idea that thymidylate synthase and dihydrofolate reductase are essential T4 baseplate components and antigenic determinants of phage particles.

2. Materials and Methods

1). Cell and Bacteriophage Strains

Escherichia coli strain B (wild-type) and wild-type phage strain T4D have been maintained in this laboratory for some time. Mutant strains bearing deletions between genes 63 and 32 were obtained from Dr. T. Homyk, Jr. (Homyk and Weil, 1974), Vanderbilt University; these included *del*7 and *del*9, the objects of this study. The *frd* gene recombinant plasmid pSP19 was constructed in this laboratory (Purohit et al., 1981). *E. coli* MB151, a strain containing the *td* gene recombinant plasmid pKTd, was obtained from Dr. Marlene Belfort, New York State Department of Health (Belfort et al., 1983).

2). Purification of T4 Phage DNA

The phage particles in a crude lysate were concentrated by centrifugation at 23,000 x g for 2 hours. The phage pellet was resuspended in one tenth the original volume in M9 salts solution. The suspension was centrifuged at 4,000 x g for 10 minutes to remove the remaining cellular debris. Phage particles from the supernatant were collected by centrifugation at 35,000 x g for 30 minutes and resuspended in one twentieth volume of M9 salts solution. Phage DNA was isolated from the purified phage suspension by phenol extraction followed by ethanol precipitation (Maniatis et al., 1982).

3). Southern Blotting Analysis

T4 DNA was digested with restriction enzymes and fractionated in a 0.7% agarose gel as described (Maniatis et al., 1982). The DNA was transferred to a nylon membane (Bio-Rad) and hybridized to a DNA probe that had been ^{32}P labeled by the hexamer primer methed (Feinberg and Vogelstein, 1984).

4). Western Blotting Analysis

Approximately 50 μg protein of each sample was electrophoretically fractionated in a 10% SDS/polyacrylamide gel and electro-transferred to a nitrocellulose membrane. 1:1000 dilutions of primary and secondary antibodies were used in immune reactions as described previously (Blake et al., 1984).

5). Antiserum Inactivation of Phage Infectivity

This was done as described by Capco and Mathews (1973), except that the experiment with dihydrofolate reductase antiserum was carried out with one tenth dilution of the antiserum instead of one fiftieth dilution.

6). Dihydrofolate Reductase Inactivation by Its Antiserum

50 μl of T4-infected *E. coli* cell extract was incubated with 20 μl of anti-DHFR in a 1.0-ml dihydrofolate reductase enzyme assay reaction mixture complete except for NADPH, for a period of time at room temperature. The enzyme activity remaining after antiserum

treatment was assayed as described by Mathews and Sutherland (1965).

3. Results

1). Southern Blotting Analysis of Deletion Mutants With T4 Dihydrofolate Reductase Gene Probe

The deletions in the 63-32 region of the T4 genome were originally mapped by DNA heteroduplex mapping (Homyk and Weil, 1974). The *td* gene, encoding thymidylate synthase, and the *frd* gene, encoding dihydrofolate reductase, are located in this region. The results indicated that both the *frd* gene and the *td* gene were deleted completely, or nearly so, in both *del7* and*del9*. We used Southern blotting and took advantage of the availability of cloned *frd* gene and *td* genes as probes to map the deletions more precisely.

For Southern blotting analysis, T4 genomic DNA must be digested and the fragments resolved on agarose gels. However, T4 DNA contains glucosylated hydroxymethyl-deoxcytidylate residues. This modification makes it resistant to digestion by most commonly used restriction enzymes. So far only six restriction enzymes are known to cleave modified T4 DNA (Kutter and Guttman, 1986), but some of them cannot digest it to completion. For Southern blotting analysis of the deletion mutants, we selected the restriction enzymes Eco R5 and Nde 1, based on their ability to digest unmodified DNA to near completion and to fragments of distinguishable sizes.

The Eco R5 digestion pattern is particularly useful, because there is a distinctive pattern of cleavage sites in the region between genes 63 and 32. This is shown in Figure IV-1. Three Eco R5

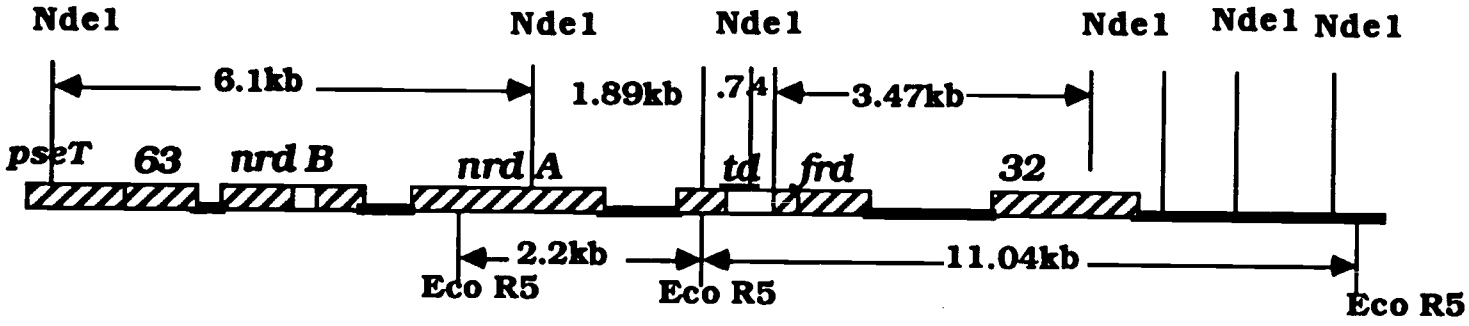


Figure IV-1. Restriction map of the gene 63 and 32 region of bacteriophage T4 DNA.

fragments cover the whole 63-32 region, and an 11.04-kb fragment contains the whole *frd* gene. Upon digestion of wild-type and deletion mutant DNAs with Eco R5 followed by electrophoresis, we observed DNA differences between wild-type and deletion mutant DNAs, as shown in Figure IV-2. The 11.04-kb fragment is absent from the deletion mutant patterns. Since *del7* and *del9* also contain a second deletion in the *rII* gene (Homyk and Weil, 1974), a 7.5-kb Eco R5 fragment that contains the *rII* cistrons was also absent from the deletion mutant DNAs.

The T4 *frd* gene has been cloned and expressed in our laboratory (Purohit et al., 1981; Purohit and Mathews, 1984). A 1.1-kb Hind III fragment containing the *frd* gene was excised from the recombinant plasmid pSP19 and used as probe for Southern blotting analysis (Figure IV-3). When Eco R5-digested T4 DNA was hybridized with the radioactive labeled *frd* gene probe, the probe hybridized to the wild-type 11.04-kb DNA fragment but not to any fragment in *del7* and *del9* DNA. The probe also hybridized a higher-molecular-weight band, probably resulting from incomplete digestion of T4D DNA. In any case, this experiment shows that the *frd* gene is totally deleted in mutants *del7* and *del9*.

In order to more precisely map the deletions, we used another restriction enzyme, Nde 1. One problem with this enzyme is that it digests T4 DNA into so many fragments that the difference between wild-type and mutant T4 DNAs cannot be observed on an ethidium bromide-stained agarose gel. Wild-type and mutant T4 DNAs were

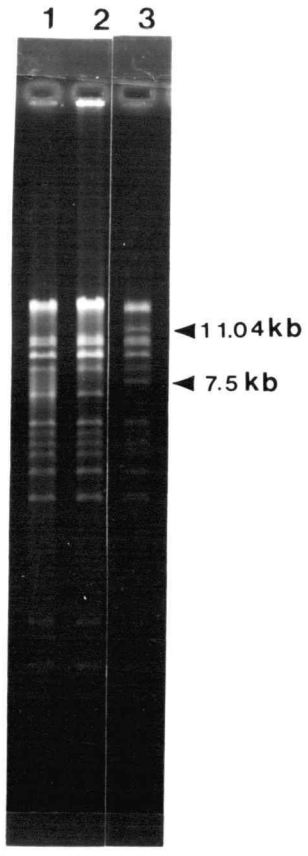


Figure IV-2. Eco R5-digested T4 DNAs fractionated on a 0.7% agarose gel. Lane 1, del7 DNA; lane 2, del9 DNA; and lane 3, T4D DNA stained with ethidium bromide.

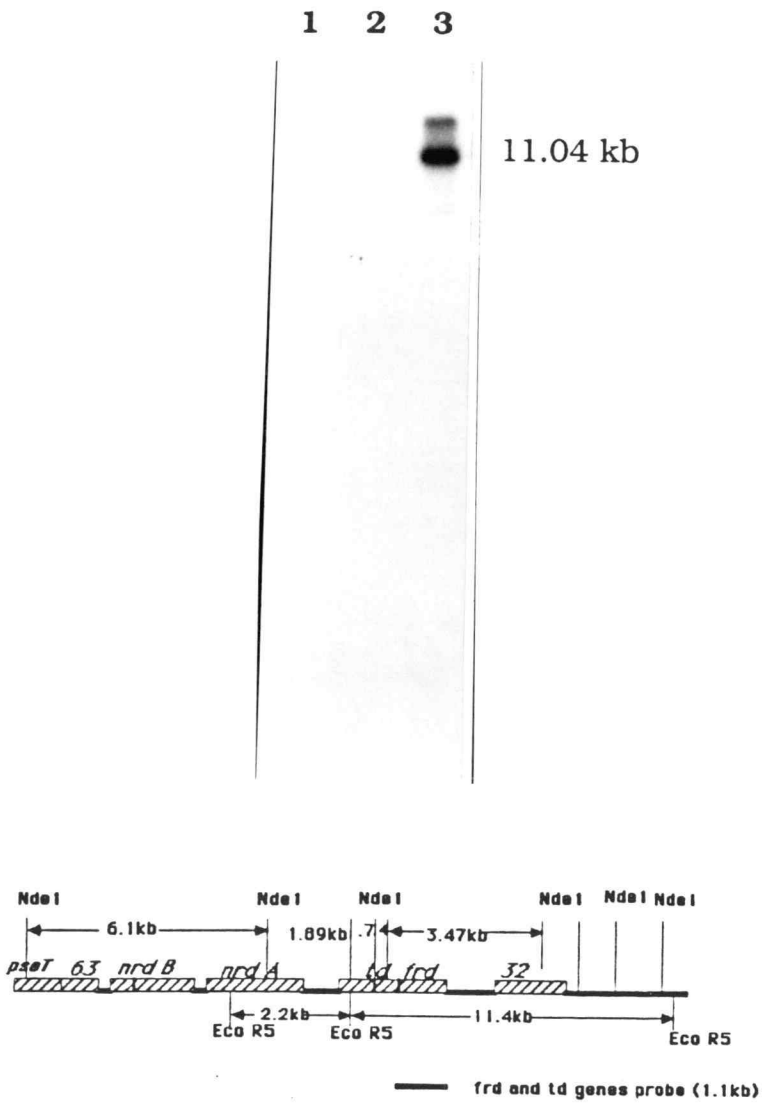


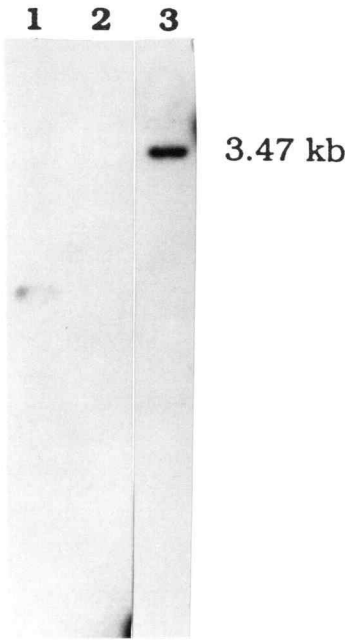
Figure IV-3. Southern blotting analysis of Eco R5-digested T4 DNAs with the *frd* gene probe. Eco R5-digested T4 DNAs were separated on an agarose gel and transferred to a nylon membrane. A hexamer primer-labeled *frd* gene probe, shown in the lower part of the figure, was used for hybridization. Lane 1, *del* 7 DNA; lane 2, *del* 9 DNA; and lane 3, T4D DNA.

digested with Nde 1 and hybridized with the same radiolabeled *frd* gene probe after transfer to a nylon membrane. According to the Nde 1 restriction map in Figure IV-1, the *frd* gene lies in a 3.47-kb DNA fragment in wild-type T4 DNA. As expected, Figure IV-4 shows that the *frd* gene probe hybridized to a fragment of the correct size in wild-type T4 DNA. Again, nothing was hybridized by the *frd* gene probe in the deletion mutant DNAs.

2). Southern Blotting Analysis of Deletion Mutants With T4 Thymidylate Synthase Gene Probe

To see if the *td* gene is also deleted in the mutants *del7* and *del9*, we used a *td* gene probe to hybridize the same membrane onto which Nde 1-digested DNA was transferred. The 1.6-kb *td* gene probe was from a recombinant plasmid pKTd (Belfort et al., 1983), and it contains the whole *td* gene and a small portion of the *frd* gene. As shown in Figure IV-5, the 1.6-kb DNA fragment contains four Nde 1 restriction fragments in wild-type T4 DNA. The autoradiogram shows what we expected for wild-type T4 DNA: four DNA fragments of the right size were hybridized by the *td* gene probe. It also shows that nothing in *del7* or *del9* DNA was hybridized by the *td* gene probe, which means that not only the *frd* gene but also the *td* gene is completely deleted in these mutants.

3). Southern Blotting Analysis with a 5.2-kb T4 DNA Fragment To Precisely Map the Ends of the Deletions



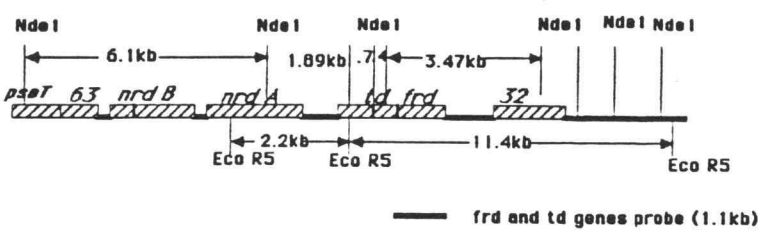


Figure IV-4. Southern blotting analysis of Nde I-digested T4 DNAs with the *frd* gene probe. Nde I-digested T4 DNAs were fractionated on a 0.7% agarose gel and transferred to a nylon membrane. The transferrd DNAs were hybridized with a radiolabeled *frd* gene probe. Lane 1, *del* 7 DNA; lane 2, *del* 9 DNA; and lane 3, T4D DNA.

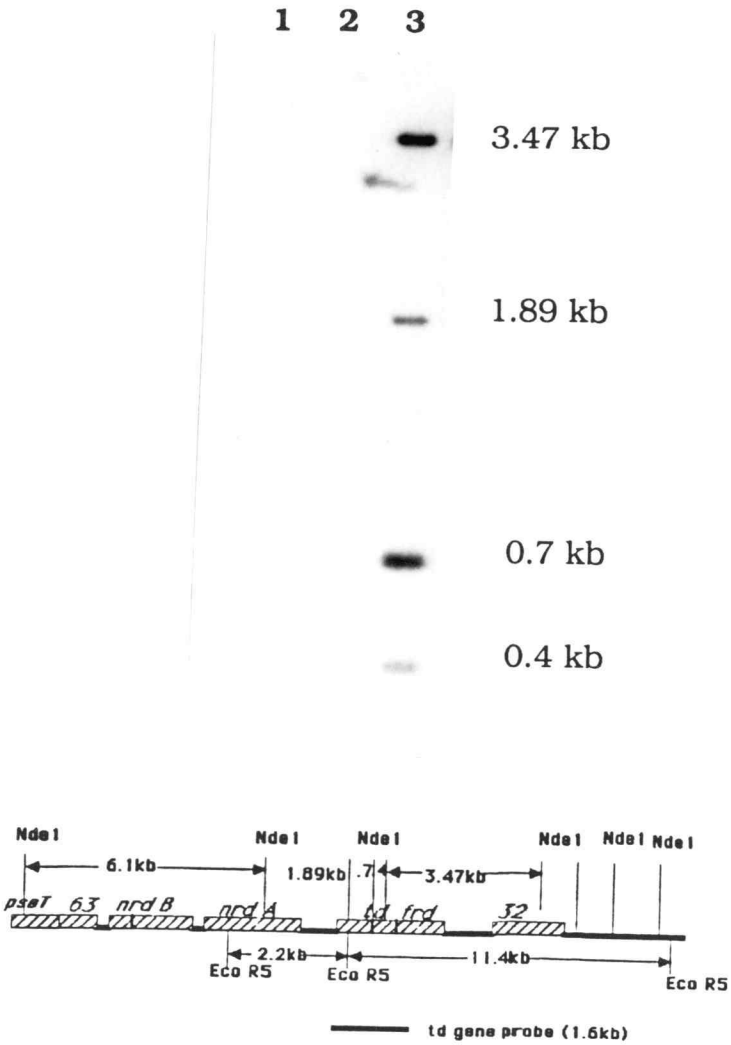


Figure IV-5. Southern blotting analysis of Nde I-digested T4 DNAs with a 1.6-kb *td* gene probe. Nde I-digested T4 DNAs immobilized on a nylon membrane were hybridized with the 1.6-kb Eco R5/Hind III DNA fragment, shown in the lower part of the figure. Lane 1, *del* 7 DNA; lane 2, *del* 9 DNA; and lane 3, T4D DNA.

A 5.2-kb Hind III DNA fragment used to map the deletion ends extends from the middle of the *td* gene to the middle of the *nrd B* gene, which codes for the small subunit of T4 ribonucleotide reductase. There are five Nde 1 restriction fragments in this 5.2-kb fragment, acccording to the restriction map of wild-type phage DNA shown in Figure IV-6. When the same Nde 1-digested T4 DNA immobilized on the membrane was hybridized with the 5.2-kb DNA probe, five bands were seen in wild-type T4 DNA, as expected (Figure IV-6). In the deletion mutants, a 6.1-kb fragment was hybridized similarly to the one seen in wild-type DNA. In addition, a new 2.5-kb fragment was hybridized in the deletion mutants. The new fragment is generated by joining two partially deleted Nde 1 fragment remainders in the mutant DNA. By combining results of Southern blotting experiments with all of the different probes, the left end of the deletion was mapped to the amino terminal end of the *nrd*A gene, which encodes the large subunit of T4 ribonucleotide reductase, and the right end of the deletion was mapped to a site immediately upstream of the *frd* gene. The deletion is 4.0 kb in length, shown in Figure IV-7.

4). Characterization of the Deletion Mutants at the Protein Level.

We carried out Western blotting analysis with antisera to T4 thmidylate synthase and dihydrofolate reductase purified from *E. coli* strains carrying cloned genes. Neither anti-TS nor anti-DHFR reacted with corresponding proteins in the deletion mutants, as shown in Figure IV-8. Enzymatic assays were carried out with deletion mutant-

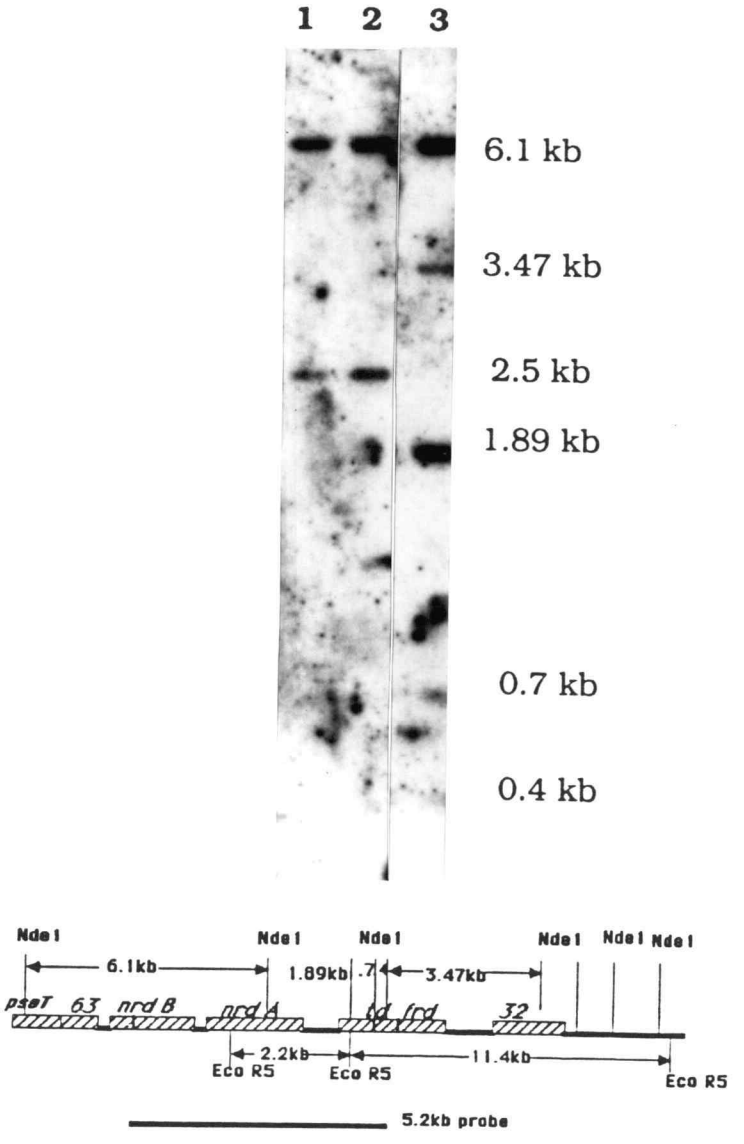


Figure IV-6. Mapping of the deletion ends with a 5.2-kb Hind III T4 DNA fragment. The map of the 5.2-kb Hind III fragment is shown in the lower part of the figure. Nde I-digested T4 DNAs immobilized on a nylon membrane were hybridized with the radiolabeled 5.2-kb fragment and exposed to autoradiography. Lane 1, *del* 7 DNA; lane 2, *del* 9 DNA; and lane 3, T4D DNA.

4.0-kb

Deletion in *del 7* and *del 9*

Figure IV-7. Deletion in 63-32 region of *del 7 and del 9* DNA, mapped by Southern blotting .

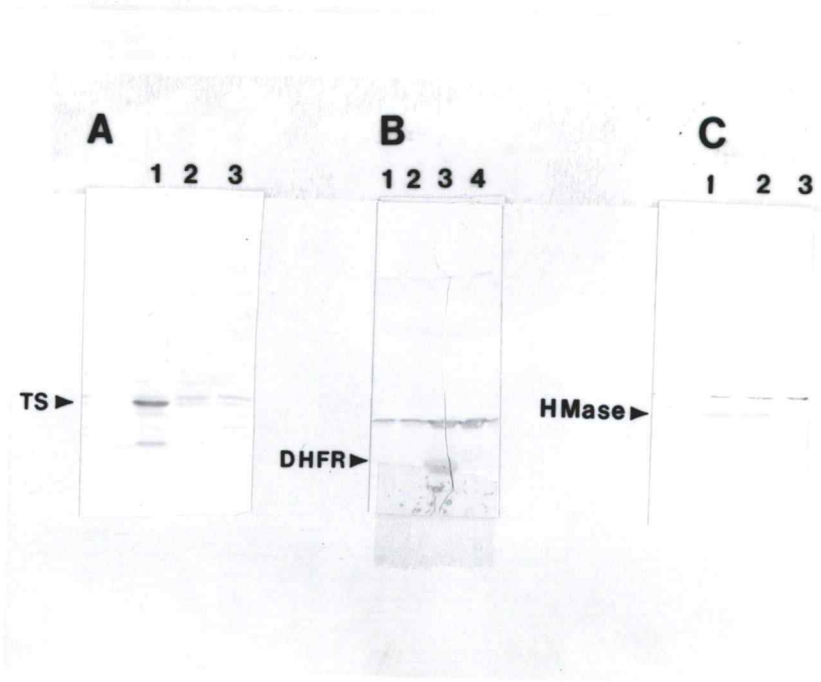


Figure IV-8. Western blotting with T4 phage-infected *E. coli* cell extracts, anti-DHFR and anti-TS. Panel A, immune reaction with anti-TS; panel B, immune reaction with anti- DHFR; panel C, immune reaction with anti-dCMP hydroxymethylase as control to ensure that infection had occured in all cells. The cell extract applied to each lane of panel A and C: lane 1, T4D-infected cell extract; lane 2, *del* 7-infected cell extract; lane 3, uninfected cell extract. The cell extract applied to each lane of panel B: lane 1, *del* 7-infected cell extract; lane 2, *del* 9-infected cell extract; lane 3, T4D-infected cell extract; and lane 4, uninfected *E. coli* cell extract. The proteins were identified by their molecular weight, shown by arrows.

infected cell extracts. Figure IV-9 confirms our earlier observation that no T4-induced thymidylate synthase or dihydrofolate reductase activities can be detected in the mutant-infected cell extracts.

5). Phage Infectivity Neutralization Experiments With Deletion Mutants and Antisera Against Cloned T4 Gene Products

Our previous studies showed that the infectivity of phages bearing deletions in the 63-32 region can be neutralized by antisera against T4 thymidylate synthase or dihydrofolate reductase, and that mutant-infected cell extracts contain proteins that cross-react with both antisera (Capco and Mathews, 1973; Mosher et al., 1977). These results were difficult to reconcile with the apparent absence of *td* and *frd* genes in these strains. One possible explanation was that the *frd* gene and *td* gene were not totally deleted, and truncated proteins could still be produced and incorporated into the baseplate in the deletion mutants(Capco and Mathews, 1973; Mosher et al., 1977). Our data here show that this is not possible; both the *td* gene and the *frd* gene are completely absent from the mutant DNAs. The current results made us suspect the antisera used in the early studies. Even though the antisera were generated from electrophoretically homogeneous proteins from T4-infected cells, and even though immunodiffusion experiments suggested the presence of antibodies to single proteins, we could not eliminate the possibility that trace amounts of other T4-induced protein contaminants also raise antibodies in the sera. In other words, the antibody which neutralizes

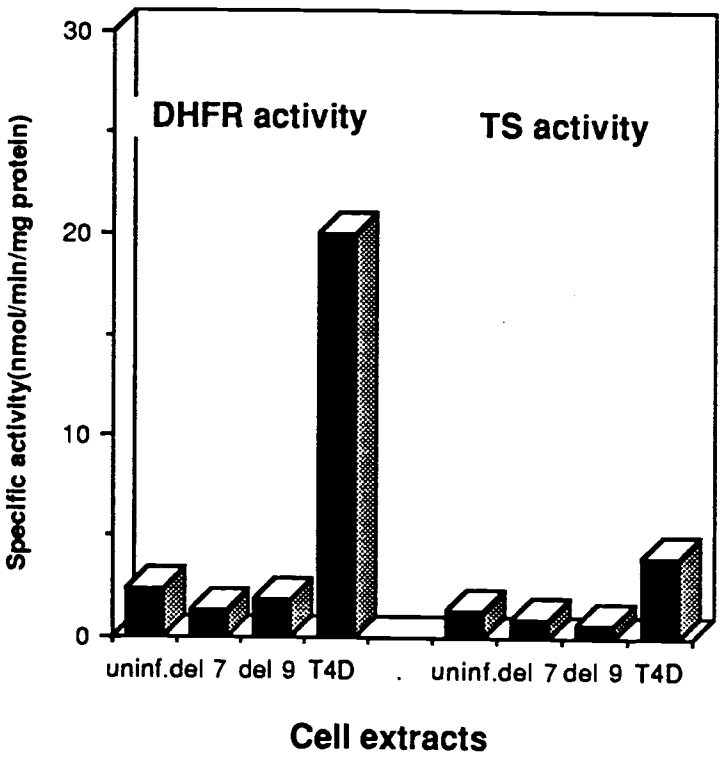


Figure IV-9. Dihydrofolate reductase and thymidylate synthase enzyme assays with wild type and deletion mutant-infected *E. coli* cell extracts. 50 µl of each cell extract was used as enzyme source, and the assays were run as described previouly (Mathews and Sutherland,1965; Mathews and Cohen, 1963).

phage infectivity might not react against dihydrofolate reductase or thymidylate synthase but some other T4 proteins in the old antisera.

Accordingly, we made new antisera against T4 dihydrofolate reductase and thymidylate synthase. The proteins used to make new antibodies were from cloned T4 gene products, not from T4-infected *E. coli* cells. Even though the proteins purified from cells containing cloned T4 genes may be contaminated with trace amount of *E. coli* host proteins, the preparations cannot contain any other T4 proteins.

With the newly generated antibodies, we carried out phage infectivity neutralization experiments of the kind carried out a decade ago in our laboratory. If thymidylate synthase and dihudrofolate reductase are the baseplate components that serve as antigenic determinants of phage particles, antibodies against the cloned gene products should be able to neutralize infectivity of wild-type T4 phage but not that of the deletion mutants. Figure IV-10 shows the result of the experiment. Unexpectedly, dihydrofolate reductase antiserum not only failed to neutralize infectivity of deletion mutants, but also that of T4D. We observed the same result with thymidylate synthase antiserum (data not shown). To optimize experimental conditions for phage infectivity neutralization, we have tried different phage dilutions as well as different antiserum dilutions. We did not see any neutralization activity in those conditions.

To further test our experimental conditions, our old dihydrofolate reductase antiserum was used as a control. Figure IV-11 shows that the old antiserum has retained some ability to neutralize phage

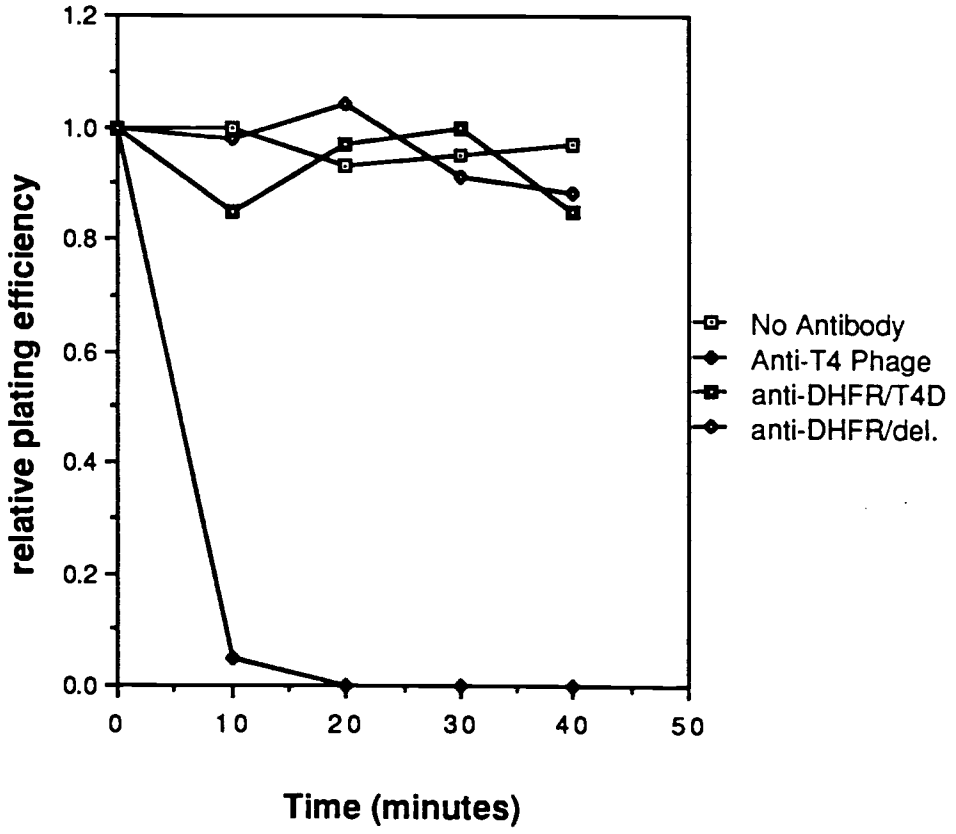


Figure IV-10. Neutralization of T4 phage infectivity by antiserum against dihydrofolate reductase. 10^5 phages were incubated with 1:10 dilution of antiserum for the indicated time intervals at 37 C. The surviving phages were diluted 1:1000 and plated. Each data point represents an average value obtained from two separate experiments.

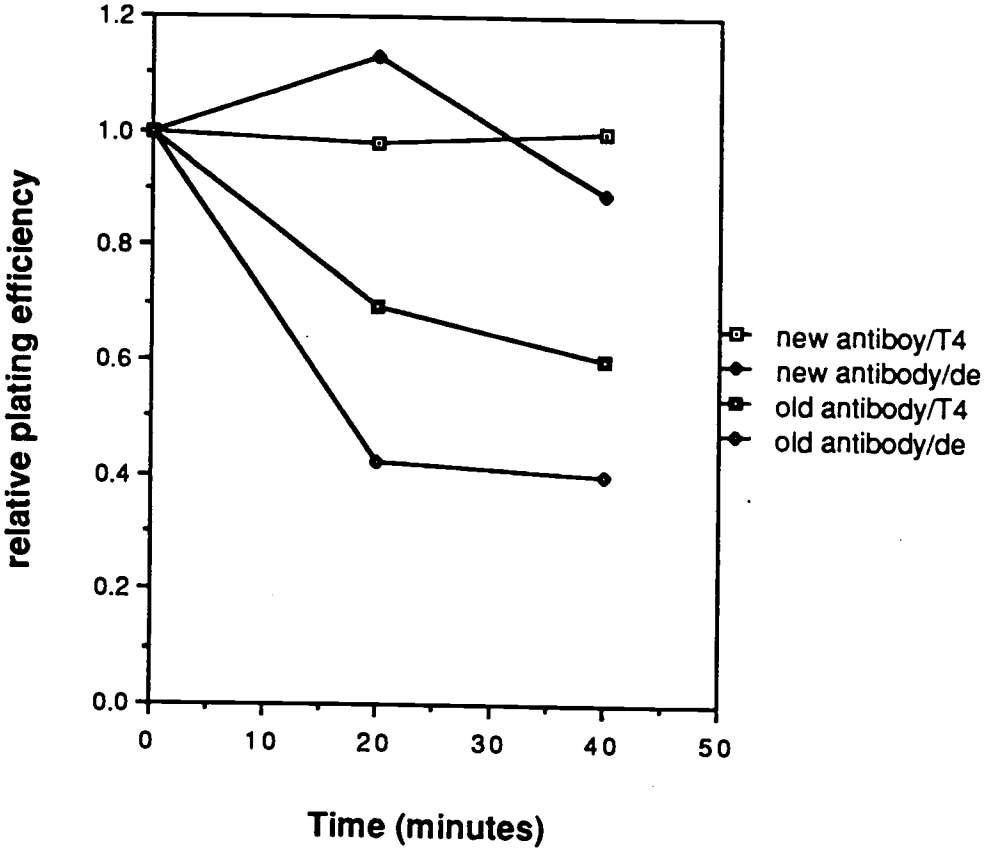


Figure IV-11. Comparison of the neutralization activities of new anti DHFR serum with old anti-DHFR serum.

infectivity, and that *del7* is more sensitive than T4D, but under the same experimental conditions the new antiserum contains no phage-neutralizing activity. Although unlikely, we must consider the possibility that the old antiserum contains a higher concentration of DHFR-specific antibodies than does the new antiserum, so that we can only observe the neutralization activity of old antiserum. To evaluate this idea, we tested the ability of each antiserum to inhibit enzyme activity. However, Figure IV-12 shows that within the limits of our experimental conditions the two antisera are equal in terms of their ability to inactivate dihydrofolate reductase enzyme activity.

6). Biological Properties of *del7*

As noted earlier, the *frd* and *td* gene products were shown to be structural elements of the T4 phage baseplate, in work both by Kozloff and colleagues and from our laboratory (Capco and Mathews, 1973; Kozloff et al., 1975; Kozloff et al., 1975; Kozloff et al., 1977; Kozloff et al., 1970; Mathews, 1971; Mathews et al., 1973; and Mosher et al., 1977). The viability of deletion mutants that completely lack these genes and their products calls into question whether the two proteins play essential roles as baseplate components. To be sure, phages containing these deletions grow quite poorly (Homyk and Weil, 1974). We have not carried out extensive biochemical analyses of the defective phenotypes associated with these deletions, for two reasons: (1) the phages grow so poorly that it is difficult to prepare lysates of sufficiently high titer for biochemical experiments; (2) because dihydrofolate reductase and thymidylate synthase both participate in

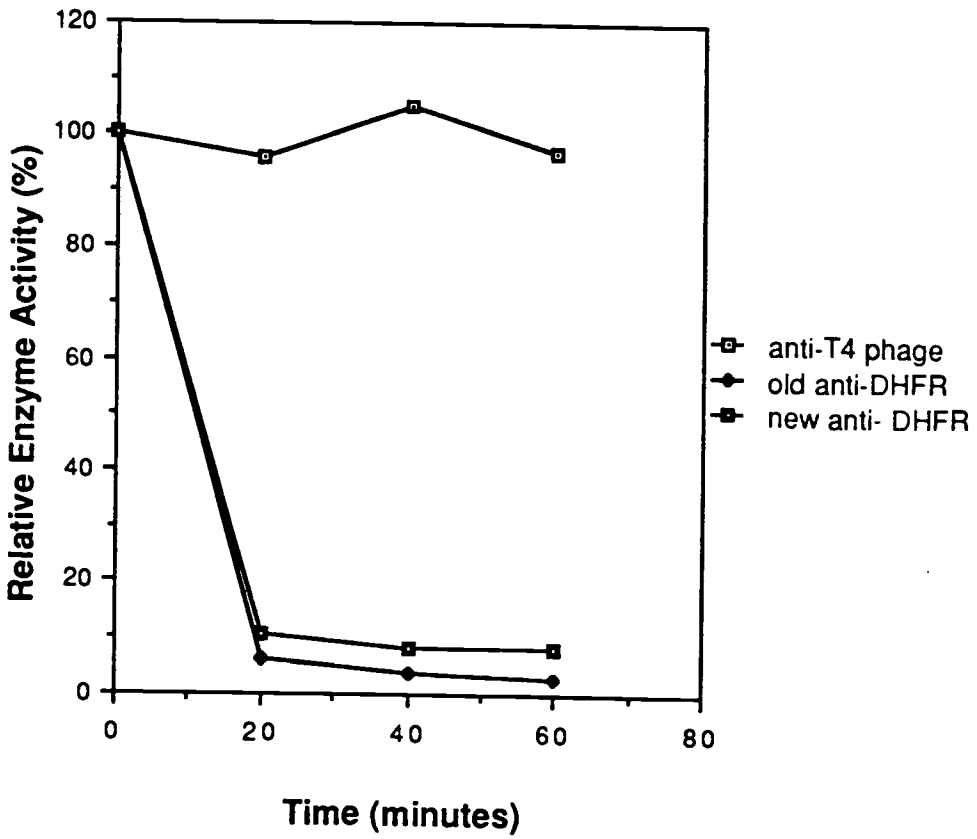


Figure IV-12. Inactivation of T4 dihydrofolate reductase by its antisera. 50 µl of each phage-infected *E. coli* cell extract was incubated with 20 µl anti-DHFR in an one ml dihydrofolate reductase enzyme assay reaction mixture except NADPH for the indicated time intervals at room temperature. The remaining enzyme activity after phage inactivation was assayed as described by Mathews and Sutherland (1965).

DNA synthesis (Mathews, 1967; Mathews, 1966; Mathews, 1965), it may be difficult deciding whether a defective phenotype results from defective DNA metabolism or baseplate function. Nevertheless, we have carried out a few preliminary biological experiments. Figure IV-13 shows that *del7* adsorbs to its host at the same rate as T4D, although measurements of DNA injection might be a more sensitive indicator of baseplate function. Also we have determined phage burst sizes, shown in Table IV-1. *del* 7 is severely restricted in its growth on *E. coli* B. Since *del* 7 contains the rII deletion, r1589, comparison with T4r1589 is more appropriate than comparison with T4D. While *del* 7 does grow very poorly on *E. coli* B, the restriction of growth is much less severe in infection of a host strain carrying a plasmid pPS2, which overproduce *E. coli* ribonucleotide reductase by at lease tenfold (Sargent et al., 1989.). This host was used in an attempt to overcome the defect in deoxyribonucleotide metabolism imposed by the *nrdA*, *frd*, and *td* deletions. Since wild-type phage growth is somewhat restricted in this host, we interpret the data to mean that the poor growth of *del* 7 is partly, but not completely, bypassed when the block to deoxyribonucleotide synthesis is circumvented. The results suggest that *del* 7 (and *del* 9) are impaired in their growth by a factor one and above the limitation in deoxyribonucleotide synthesis. We suggest that factor is the structural role played by T4 dihydrofolate reductase and thymidylate synthase. While essential for maximum reproductive success of the virus, these structural enzymes are apparently not critical for phage viability.

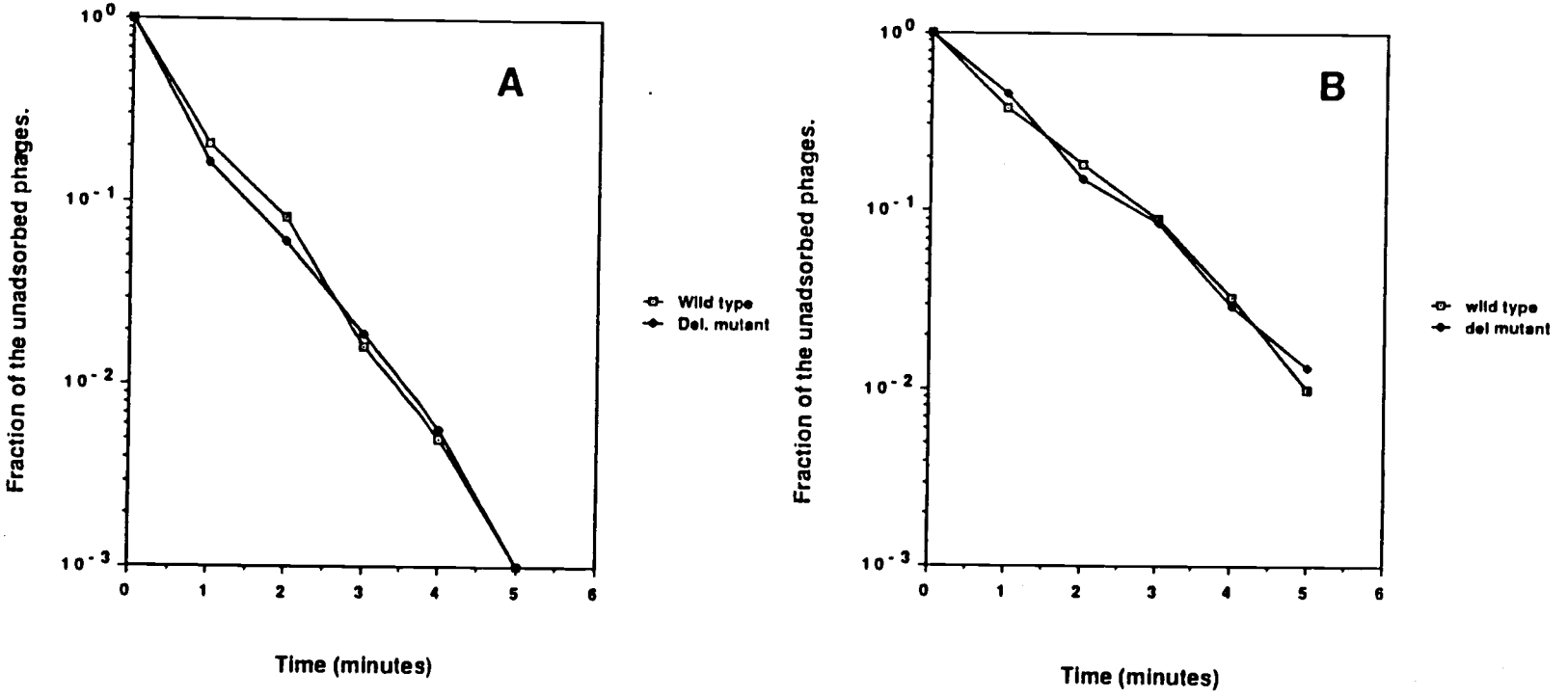


Figure IV-13. Adsorption of T4 phage to its host. T4D or *del 7* mutant phages were added to an *E. coli* cell culture, gorwn to a density of 2 X 10^8 cells/ml, at a multiplicity of 1 or 10. Every minute after addition of the phages, 100 µl of the cell culture was withdrawn, and *E. coli* cells were inactivated by chloroform treatment. The unadsorbed phages remaining in the supernatant were diluted and plated in duplicate. Panel A and B show phage adsorption kinetics to *E. coli* B cells at multiplicities of 1 and 10, respectively.

Table IV-1. Phage Burst Sizes

Phage strain	*E. coli* strain	Phage yield, pfu/cell
T4D	B	527
T4 r1589	B	171
T4 *del* 7	B	10
T4D	pPS2/ED8689	109
T4 r1589	pPS2/ED8689	121
T4 *del* 7	pPS2/ED8689	51

Phage yields were determined under lysis-inhibited conditions previously described (20). The burst sizes on *E. coli* B are averages of five independent determinations, and the other burst sizes result from triplicate determinations.

4. Discussion

Our early attempts to define the roles of T4 thymidylate synthase and dihydrofolate reductase as virion components led to a paradoxical result: phages reported to be deleted for these genes produced proteins that cross-reacted with antisera against electrophoretically homogeneous preparations of both enzymes (Capco and Mathews, 1973; Mathews et al., 1973; Mosher et al., 1977; and Mosher and Mathews, 1979). It was timely for us to reinvestigate this apparent paradox, using information and reagents that have become available in the ten years since we last studied this problem -- a restriction map of this region of the T4 genome, cloned fragments to use as hybridization probes, and cloned T4 *td* and *frd* genes, which allow purification of the respective enzymes totally free of contamination by other phage-coded proteins. Using these resources, we have confirmed the original report of Homyk and Weil (Homyk and Weil, 1974) that *del7* and *del9* each carries a deletion of about 4 kb, which totally eliminates the *td* and *frd* gene products. These phages are not targets for neutralization by antisera against either dihydrofolate reductase or thymidylate synthase prepared from cloned genes. Since these newly prepared antisera also fail to neutralize the infectivity of T4D, the conclusion seems inescapable that the phage-neutralizing components in our old antisera were antibodies against minor components of our purified enzyme preparations, components that could not be detected by techniques available at the time.

What, then, are the roles of thymidylate synthase and dihydrofolate reductase as virion proteins? The experiments described here do not affect the interpretation of other evidence from the Kozloff and Mathews laboratories, including enzyme activity measurements, which do locate small amounts of each protein in the T4 tail baseplate (Kozloff et al., 1975; Kozloff et al., 1977; Kozloff et al., 1970; and Mosher et al., 1977). The extremely poor growth of the deletion mutants in wild-type strains of *E. coli* suggests that the roles played by these proteins, while not essential for phage viability, are quantitatively important. Now that the deletions in *del7* and *del9* have been characterized, and now that the purified gene products are available in quantity from cloned *td* and *frd* genes, we have a good opportunity to define the functions of these proteins as structural elements.

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