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Title: Mutagenic Mechanisms Associated with Perturbations of DNA Precursor Biosynthesis in Phage T4

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A crucial factor in determining the accuracy of DNA replication is maintenance of a balanced supply of deoxyribonucleoside triphosphates (dNTPs) at replication forks. Perturbation of dNTP biosynthesis can induce dNTP pool imbalance with deleterious genetic consequences, including increased mutagenesis, recombination, chromosomal abnormalities and cell death. Using the T4 bacteriophage system, I investigated the molecular basis of mutations induced by imbalanced dNTP pools in vivo. Two approaches were adopted to disturb dNTP biosynthesis: 1) using mutations which affect the deoxyribonucleotide biosynthesis pathway; 2) exogenously supplying mutagenic deoxyribonucleoside analogs which are then taken up by cells and are metabolized to dNTPs. The levels of dNTPs under different conditions were measured in crude extracts of phage-infected cells, while mutagenic effects were quantitated by analysis of certain *rII* mutations, thought to revert to wild type along either GC-to-AT or AT-to-GC

transition pathways. The mutation pathways stimulated by dNTP pool perturbations were confirmed by direct DNA sequencing after amplification of template by the polymerase chain reaction (PCR).

By replacing phage ribonucleotide (rNDP) reductase with the host, *Escherichia coli*, rNDP reductase, in phage-infected cells, I examined the mechanism of mutation induced by the thymidine analog 5-bromodeoxyuridine (BrdUrd) in vivo. Although both AT-to-GC and GC-to-AT transition mutations were stimulated many hundred-fold when cells were grown in medium containing 100 μM BrdUrd, GC-to-AT transitions were stimulated predominantly when T4 reductase was active, while AT-to-GC transitions were stimulated more when *E. coli* reductase was active. By examining the control by dNTPs on CDP reduction, I found that the T4 rNDP reductase is substantially inhibited by either BrdUTP or dTTP in crude enzyme extracts. These experimental results are consistent with the hypothesis that mutagenic effects of BrdUrd are based on dNTP perturbations, supporting the model that rNDP reductase is a major determinant of BrdUrd mutagenesis.

I also studied the mutator phenotype of one temperature-sensitive conditional lethal mutant, T4 *ts* LB3, which specifies a thermolabile T4 deoxycytidylate (dCMP) hydroxymethylase. At the sites of different *rII* mutations, I found 8- to 80-fold stimulation of GC-to-AT transitions induced by *ts* LB3 at a semipermissive temperature (34^{o} C). Sequence analysis of revertants from the most sensitive gene marker, *rII* SN103, showed that either cytosine within the mutated triplet can undergo change to either thymidine or adenine, supporting a model in which mutagenesis induced by *ts* LB3 at a semipermissive temperature is based

on dNTP pool perturbations. The putative depletion of hydroxymethyl-deoxycytidine triphosphate (hm-dCTP) caused by the temperature-labile dCMP hydroxymethylase presumably enlarges effective dTTP/hm-dCTP and dATP/hm-dCTP pool ratios, resulting in the observed C-to-T transition and C-to-A transversion mutations. However, no significant dNTP pool abnormalities were observed in extracts from *ts* LB3 phage-infected cells even when cells were grown at the semi-permissive temperature, suggesting that imbalanced dNTP pools occurred only locally, close to replication forks. These results support a model of dNTP "functional compartmentation", in which DNA replication is fed by a small and rapidly depleted pool, with the bulk of measurable dNTP in a cell representing a replication-inactive pool.

To further characterize the mutagenic specificity and DNA site specificity induced by T4 *ts* LB3, I developed a fast forward mutation approach using thymidine kinase as a marker gene. The studies confirmed that the principal mutagenic effect induced by *ts* LB3 is C-to-T transition, while C-to-A transversion mutagenesis also occurs. Analysis of DNA sequences around each mutation also suggests that local DNA context influences mutation frequency.

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Chapter I

General Introduction

Organisms can maintain their genetic identity only if the genetic material, DNA, is replicated faithfully. DNA replication fidelity is determined by all the varied activities associated with DNA metabolism and its environment during the cell cycle (Loeb and Kunkel 1982, Reichard 1988). In particular, on the basis of very general kinetic considerations, based ultimately on the law of mass action, it is clear that the fidelity of DNA synthesis must depend on the concentrations of dNTP substrates (Goodman 1988). Extensive studies have shown that imbalanced dNTP pools reduce replication fidelity in vitro and induce mutations in vivo (Kunkel 1988, Kunkel and Bebenek 1988, Kunz 1988, MacPhee et al 1988, Meuth 1989). Therefore, knowledge of the molecular effects of dNTP levels on genetic stability is not only important for an understanding of basic molecular genetics, but it appears also to be central for effective design and use of antimetabolites to prevent or treat cancer and other genetic diseases as well as viral and other microbial infections.

It is obvious that the mutations observed represent the overall results of DNA replication, in which three steps can be differentiated: a misincorporation step, an editing step and a mismatch repair step.

Mutation can also occur during repair, such as error-prone repair after UV damage. Perturbations of dNTP pools can cause misincorporation of the nucleotide in excess due to competition between correct and incorrect nucleotides at a DNA biosynthetic site. Misincorporation may be exacerbated by the interference of excess precursor with proofreading functions of the replication complex. It is also possible that pool imbalances may provide a positive or negative signal for repair systems. Meanwhile, some chemical mutagens may act not by direct interference with DNA replication, but instead by affecting DNA precursor biosynthesis, causing dNTP pool imbalance. To distinguish among these various mechanisms, an understanding of the nature of mutagenesis induced by perturbation of DNA precursor biosynthesis at the nucleotide level is essential.

This dissertation is concerned with mechanisms of mutation induced by imbalanced dNTP pools in vivo. The goal of the research presented here is to develop a system in which dNTP pools are disturbed either by altered DNA biosynthesis pathways or by exogenous supply of mutagens; to study quantitatively the relationship between disturbed dNTP pools and mutagenesis; and to investigate DNA site specificity and mutagenic specificity in defined genetic markers. Specifically, I chose the T4 phage-coded rNDP reductase and dCMP hydroxymethylase as determinant proteins to study the molecular bases of mutagenesis induced by perturbations of dNTP pools in T4 bacteriophage.

1. Bacteriophage T4 biology

T4 is a large bacterial virus, which infects *E. coli*, with an icosahedral head filled with double-strand DNA and a complex tail through which the DNA is extruded during infection. The genome of T4 contains about 166 kbp with 3% terminal redundancy, formed from concatenated replication intermediates. About 90% of the genome has been sequenced, with nearly 200 virus-encoded products having been identified. A detailed T4 genetic map is shown in Figure I-1.

Many features of the T4 life cycle are common to infections by large DNA viruses. The virion is merely a vehicle for conveying the viral genome to a host cell. As soon as the viral DNA is injected into the cell through the viral tail, the host RNA polymerase begins to transcribe a series of viral early genes, such as nucleases for degradation of host DNA and enzymes for synthesis of T4 DNA. Meanwhile, all synthesis of host proteins and mRNA ceases promptly. Most T4 early proteins involved in nucleotide and nucleic acid metabolism are transcribed by ADP-ribosyl-modified host RNA polymerase. Early gene transcription ceases at about 12 minutes after infection at 37^{0} C. T4 DNA replication begins at 5 minutes after infection, being initiated either by modified host RNA polymerase at distinct replication origins or by phage DNA recombination (Mosig 1983). T4 late genes are transcribed by more extensively modified host RNA polymerase, along with phage encoded proteins, gp33, gp45 and gp55 (gp, gene product). The late genes encode virion structural components and enzymes for phage assembly. The onset of phage DNA replication is necessary for late gene

expression (Rabussay 1983, Geiduschek et al 1983). Three T4-encoded DNA polymerase accessory proteins have been shown to stimulate transcription at T4 late promoters in an ATP hydrolysis-requiring process (Herendeen et al 1989). The whole process from infection to lysis takes about 25 to 30 minutes, in which time about 200 phage particles are released from one infected cell. The schematic diagram of the T4 life cycle is shown in Figure I-2.

2. T4 dNTP biosynthesis

T4 infection dramatically alters the flow of precursors into nucleic acid. The DNA synthesis rate in T4 infected cells is about 10-fold higher than in uninfected *E. coli*. This is accomplished by phage-encoded proteins as well as host enzymes, for dNTP biosynthesis through both de novo and salvage pathways (Mathews and Allen 1983). By expressing its own dNTP biosynthetic enzymes immediately after infection, T4 re-orients and enhances the pre-existing host dNTP de novo pathway. Some phage-encoded proteins have identical enzymatic activities with host proteins but distinct physical and allosteric properties, while others are specific phage proteins without host counterparts. T4 also encodes enzymes to digest the host chromosome, thereby enhancing salvage pathways, for reutilizing nucleotides released from host cell DNA. In T4-infected cells, amounts of dNTPs from the degradation of host DNA through this salvage pathway are enough to synthesize more than 20 phage DNA molecules (Mathews and Allen 1983, Snustad et al 1983). The overall enzymes and reaction steps involved in T4 dNTP biosynthesis are shown in Figure I-3.

The most significant difference in dNTP biosynthesis between T4 and its host, *E. coli*, is that T4 degrades dCTP to dCMP and synthesizes 5-hydroxymethyldeoxycytidylate for DNA replication (Flaks and Cohen 1959, Wiberg et al 1962). This modification allows the phage-encoded nucleases to digest the host chromosome while protecting its own. The key enzyme involved in this modification is T4 dCMP hydroxymethylase, encoded by gene 42, which converts dCMP to 5-hydroxymethyl-deoxycytidine monophosphate (hm-dCMP), which is further phosphorylated by kinases to form hm-dCTP for DNA replication.

3. Ribonucleotide reductase as a key protein in dNTP pool regulation

As an enzyme catalyzing the first committed step in the biosynthesis of all four deoxyribonucleotides, and with allosteric regulation properties, ribonucleotide reductase is a key protein in dNTP pool regulation (Reichard 1988, Mathews 1988, 1989). The protein is a heterotetramer in most organisms, consisting of two large subunits and two small subunits. The schematic model of ribonucleotide reductase from *E. coli* is shown in Figure I-4. The catalytic site involves residues from both large and small subunits. The tyrosyl free radical is from the small subunit, which is stabilized by an adjacent dinuclear iron center (Lynch et al 1989, Nordlund et al 1990) and believed to initiate the radical-based reaction (Reichard 1988). The electrons for the reduction are from redox-active cysteines on the large subunits, resulting in a direct replacement of hydroxyl group at the 2' position of the ribosyl

moiety with hydrogen.

The large subunit also contains two classes of allosteric sites: 1) substrate specificity sites, in which binding of a certain nucleotide promotes or inhibits reduction of a particular rNDP substrate, and 2) activity sites in which binding of one effector regulates reduction of all four rNDPs. Such allosteric properties are best understood in the *E. coli* enzyme. The activity site binds dATP as negative effector with low affinity (K_d = 0.1-0.5 μM), and it also binds with ATP as positive effector, regulating the overall activity of the enzyme. Photoaffinity labeling experiments with large subunits suggested that the region around cysteine-229 is responsible for the regulation of substrate specificity (Eriksson et al 1986). The specificity site binds dATP with high affinity (K_d = 0.03 μM), and it also binds with ATP, dGTP and dTTP. Binding of a certain nucleotide at either an activity site or a specificity site apparently changes the protein conformation at the catalytic site, causing an altered V_{max} of reaction and K_m for a substrate (Larsson and Reichard 1966, Brown and Reichard 1969, Ehrenberg and Reichard 1972, von Döbeln and Reichard 1976, Thelander and Reichard 1979).

The major differences between *E. coli* and T4 rNDP reductase are more kinetic than structural (Berglund 1972, 1975). The T4 enzyme possesses the same subunit structure, having two polypeptides each in the large subunit and small subunit, with molecular weights of 86 kDa and 43.5 kDa, respectively. The subunit association in T4 is much stronger than that seen in host counterparts during purification. Functionally, T4 enzyme uses all four rNDP substrates and almost the

same triphosphate mediators as in *E. coli*. However, in terms of allosteric regulation properties, there are three significant differences between T4 and its host. 1) dATP is a negative activator for all four rNDP reductions in *E. coli*, while it is a positive activator for pyrimidine rNDP reduction in T4; 2) dTTP at high concentration inhibits CDP reduction in *E. coli*, but not in T4, although dTTP stimulates GDP reduction in both systems; 3) Though dCTP has no any allosteric effect on *E. coli* rNDP reductase, hm-dCTP is an activator for pyrimidine reduction on T4 rNDP reductase. Since T4 rNDP reductase apparently is not inhibited by any dNTPs on rNDP reduction, the enzyme is called feedback resistant. In contrast, *E. coli* rNDP reductase is referred to as a feedback-sensitive enzyme. However, these data were from analyses of purified protein in vitro. What happens in vivo could be different, simply because of the fact of enzyme existence as a component of complex cellular metabolic systems.

Other allosterically regulated proteins in T4 dNTP biosynthesis include dCMP deaminase and thymidine kinase, which participate in regulation of pyrimidine deoxynucleotide pools along with rNDP reductase (Mathews and Allen 1983). A summary of all of the published regulatory effects on T4 and its host, *E coli*, enzymes in dNTP biosynthesis pathway is listed in Table I-2.

4. dNTP pool compartmentation

How are dNTP pools distributed inside cells? Measurement of enzyme kinetic parameters involved in DNA replication and its precursor biosynthesis, both in vitro and in vivo, suggests that DNA

precursors are compartmentalized near replication forks. In T4-infected cells, replicative chain growth occurs at about 700 to 800 nucleotides per second. At the same time, the replication apparatus has low affinity for dNTPs, with about 250 μM of each dNTP needed to saturate replication forks in vitro, while average intracellular concentrations of dNTPs are about 100 μM each (Mathews and Sinha 1982). However, experimental data indicated that T4 DNA polymerase is saturated with dNTPs in vivo (Mathews 1976). These observations suggest that higher dNTP concentrations than the average intracellular level must be maintained during DNA replication.

In the 1970s both Mathews' and Greenberg's groups proposed that dNTP synthesis is carried out by a complex of enzymes that is integrated with the replication machinery. Such a structure would allow deoxyribonucleotides to be "channeled", or used directly at their sites of synthesis, with restricted diffusion away from replication sites. The evidence obtained with cell-free enzyme aggregates and permeabilized cells, as well as in vivo studies, suggest the existence of such a "dNTP synthetase" multienzyme complex (Tomich et al 1974, Chiu et al 1976, Wovcha et al 1976, Flanegan and Greenberg 1977, Reddy et al 1977, Reddy and Mathews 1978, Chiu et al 1982, Allen et al 1983). Recently, Moen et al (1988) isolated a 1500-kDa multienzyme complex that synthesizes dNTP from either deoxyribonucleoside monophosphates (dNMPs) or ribonucleoside diphosphates (rNDPs). Ten enzyme activities have been identified in this complex, including rNDP reductase, dCMP hydroxymethylase, thymidylate synthase, thymidine kinase and others as shown in Figure I-5. Could this complex be physically linked to the

replication apparatus, so that distal DNA precursors would be channeled directly into DNA? By using affinity chromatography, in which dCMP hydroxymethylase was immobilized on Affi-Gel, Wang (1989) has identified several dNTP biosynthesis enzymes as well as DNA replication proteins among those proteins bound to the column. However, direct physical evidence for intracellular interactions between dNTP synthetic enzymes and proteins of the replication machinery in vivo still is lacking. Therefore, the question of DNA precursor channeling in prokaryotic systems still remains open.

5. Genetic consequences of perturbation of dNTP pools

DNA replication uses precursors, the dNTPs, which are specialized for that purpose and for little else. This limited repertoire of metabolic roles makes it possible for DNA synthesis to be regulated specifically at the level of precursor formation. Experimental observations have shown that perturbation of dNTP pools has deleterious genetic consequences, as shown in Table I-1. Severe depletion of one dNTP has been shown to lead to cell death, while less extreme dNTP pool perturbations can induce different genetic abnormalities, ranging from recombinogenic effects to increased point mutations. These results suggest that synthesis of both DNA and DNA precursors are strongly coordinated in vivo (Kunz 1982, Haynes and Kunz 1988, Kunz 1988, Meuth 1989).

The earliest evidence showing mutagenic effects of perturbation of dNTP pools comes from studies of thymidine-requiring strains of *E.*

coli and T4 phage. AT-to-GC transition mutations were induced predominantly after thymidylate starvation (Neuhard and Munch-Petersen 1966; Holmes and Eisenstark 1968, Pauling 1968, Drake and Greening 1970, Bernstein et al 1972, Bresler et al 1973, Smith et al 1973). To date, mutator phenotypes have been found in strains with lesions in genes coding for dCMP deaminase, dCMP hydroxymethylase, ribonucleotide reductase, CTP synthase, deoxyuridine triphosphatase and thymidylate synthase (Williams and Drake 1977, Hochhauser and Weiss 1978, Weinberg et al 1981, Kunz and Haynes 1982, Maus et al 1984, Roguska and Gudas 1984, Trudel et al 1984, Weinberg et al 1985, Sedwick et al 1986, Sargent and Mathews 1987, Glickman et al 1988, Sargent et al 1989). Moreover, it has also been demonstrated that mutator phenotypes can be modulated by offsetting the dNTP imbalance associated with particular enzyme deficiencies (Meuth 1981, Weinberg et al 1981, 1985), and that exogenous supplies of a particular base, nucleoside or nucleotide stimulated mutations through dNTP pool perturbations (de Vries and Wallace 1982, Kunz 1982, Rossman and Stone-Wolff 1982, Eckardt et al 1983, Goncalves et al 1984, Brendel 1985, Phear et al 1987). Similarly, inhibition of particular deoxynucleotide biosynthesis by treatment with drugs has also been shown to be mutagenic or lethal (Aebersold 1979, Wurtz et al 1979, Peterson et al 1983, Hoar and Dimnik 1985).

6. Mechanisms of mutagenesis induced by pool imbalance

The simple hypothesis is that misincorporation of a nucleotide present in excess is the major mutagenesis mechanism. Competition between correctly and incorrectly base-paired nucleotides is based upon the relative concentrations of these nucleotides at an incorporation site. This hypothesis correctly accounts for the mutation type induced by depletion or expansion of specific dNTP pools in most experiments. However, the "mass action model" cannot explain the site specificity of mutations induced by dNTP pool perturbations. Studies have shown that frequency of base misincorporation is dependent on DNA sequence context, both in vitro and in vivo (Kunkel and Bebenek 1988, Meuth 1989). In one extreme example, one particular site underwent mutagenesis at a rate about one hundred-fold higher than predicted from the dNTP pool imbalance (Sargent and Mathews 1987).

Based on mathematic models of DNA polymerization and proofreading, Fersht (1979) suggested that the next nucleotide to be added in a DNA nascent strand can enhance replication error by "pushing" the polymerization complex past the error when the next nucleotide to be incorporated is present in excess, diminishing the effectiveness of the 3'-5' proofreading exonuclease. Such "next nucleotide effect" has been observed in vitro (Kunkel 1988, Kunkel and Bebenek 1988, Bebenek and Kunkel 1990). Based on analyses of the DNA sequences of mutations stimulated by a thymidylate synthase-defective mammalian cell mutant, Meuth (1989) found that T-to-C transition mutations were enhanced when the next 3' nucleotide to be

added is T, suggesting that "next nucleotide effect" also operates in vivo. However, analysis of the T-to-A transversion mutations produced by excess dCTP gave a very different picture. Mutations were found at AC sites at a frequency about equal to the prevalence of this dinucleotide in the gene. Meuth proposed that high frequency of transversion mutations observed in their experiments is due to inefficient proofreading by 3'-5' exonuclease, regardless of what the neighbor nucleotide context (Phear and Meuth 1989, Meuth 1989). Other mechanisms of mutation at various DNA context could involve the repair system, especially under dNTP pool perturbation conditions. Correlations between imbalanced dNTP pools and abnormal repair systems, such as error-prone and excision repair, have been observed in several studies (Meuth 1981, Snyder 1984, 1985, Hunting and Dresler 1985). Although repair mechanisms could associated with dNTP pool perturbations if DNA precursor imbalances acted as inducing signals for repair or positive effectors or inhibitors of repair, direct evidence has not be obtained.

7. Molecular basis of bromodeoxyuridine mutagenesis

The mutagenic effects of the thymidine (dThd) analog, 5-bromodeoxyuridine (BrdUrd) have been observed in all organisms, from viruses to bacteria to mammalian cells. The mutagenic basis of BrdUrd was thought to be due to its ability to incorporate into DNA. Based on the greater electronegativity of the Br atom of BrdUrd relative to the CH_3 group of thymine, Freese (1959) proposed a model in which BrdUrd assumes the rare enol tautomeric form more often than does

thymine. Since this tautomer can base pair with guanine (G), BrdUrd will mispair with guanine (G) more frequently than will thymine (T). Further studies have supported this "rare tautomeric forms" hypothesis (Topal and Fresco 1976, Singer and Kusmierek 1982). Other mechanisms to explain base mismatch formations were also postulated, including " ionized bases" (Lawley and Brooks 1961, 1962, Sowers et al 1987) and " wobble base pairs" (Crick 1966, Topal and Fresco 1976, Patel et al 1982). Based on studies of hydrogen bonding between mismatched bases in synthetic BrdUrd-containing oligonucleotides with high resolution NMR spectroscopy, Goodman and his colleagues have indicated that BrdUrd:G mispairs are stabilized by hydrogen bonds (Petruska and Goodman 1985, Goodman 1988). The presence of the disfavored enol tautomer was not detected. The mispairs appeared to be as ionized Watson-Crick bases in equilibrium with the wobble structure, where the BrdUrd and G are present in their favored keto forms (Kaufman 1988, Lasken and Goodman 1984, Goodman 1988). Although Freese's tautomeric shift mechanism does not appear to be involved, his proposal of two possible mechanisms for BrdUrd mutagenesis - errors of incorporation and errors of replication - still appears to be valid. Incorporation errors were thought to occur when BrdUTP mispaired with a guanine residue in replicating DNA, resulting a GC-to-AT transition. Replication errors were thought to occur when a bromouracil residue in replicating DNA mispaired with dGTP, resulting in AT-to-GC transition (Trauter et al 1962, Lasken and Goodman 1984).

The ease with which 5-bromodeoxyuridine 5'-triphosphate (BrdUTP) substitutes for deoxythymidine triphosphate (dTTP) in DNA

replication suggested that replication errors should predominate. However, experiments showed that some mammalian cell strains with extensive substitution of bromouracil for thymine in their DNA do not accumulate lethal mutations (Bick and Davidson 1974). Some strains grow even better in the BrdUrd-containing medium. Further studies showed that BrdUrd mutagenesis depends not so much on the extent of bromouracil substitution for thymine in DNA, but upon the concentration of BrdUrd in the medium when mutagenesis is taking place. These observations led Hopkins and Goodman (1980) to propose that BrdUrd mutagenizes largely by dNTP pool perturbation, resulting in misincorporation. They predicted that GC-to-AT transitions should be the dominant mutations induced by BrdUrd, through competition between BrdUTP and deoxycytidine 5'-triphosphate (dCTP) for incorporation opposite dGMP in the template. The favorable misincorporation of BrdUTP over dCTP is, as Hopkins and Goodman pointed out, facilitated by the action of Br-dUTP as a dTTP analog, allosterically inhibiting the reduction of cytidine 5'-phosphate (CDP) by ribonucleotide reductase (Thelander and Reichard 1979). Extensive studies with mammalian systems suggested that BrdUrd mutagenesis is based on dNTP pool perturbations, in which an expanded BrdUTP competes with a depleted dCTP pool, mispairing with guanine through allosteric inhibition of rNDP reductase. The predominant mutation stimulated by BrdUrd in mammalian cells has been reported to be the GC-to-AT transition, partly because the misincorporation can be reduced by exogenously adding deoxycytidine to supply a high dCTP pool (Davidson and Kaufman 1978, 1979, Ashman and Davidson 1981, Kaufman 1984, Davidson et al 1988). The AT-to-GC transition,

stimulated by BrdUrd, was also reported as a consequence of dNTP pool perturbation (Kaufman 1984, 1988). A high correlation between AT-to-GC transition and intracellular ratio of dGTP/dATP has been observed, suggesting that a high intracellular ratios of dGTP/dATP might serve to drive the mispairing of dGTP with bromouracil residues in replicating DNA. These studies support Hopkins-Goodman's model (Hopkins and Goodman 1980), suggesting that dNTP pool perturbation is a determinant of BrdUrd mutagenesis.

8. *rII* gene as a genetic marker

The investigations of mutagenic specificity and DNA site specificity rely on defined genetic markers. One of the most valuable markers in mutation research is the T4 *rII* gene, which encodes a membrane protein of still unknown function. The *rII* genes were discovered by Hershey (1946) based on plaque morphology. Rapid lysis mutants, r^-, can form large plaques relative to wild-type phage, due to the inability of r^- mutants to establish lysis inhibition (Doermann 1948), leading to more cycles of infection and lysis than undergone by wild-type phage in the same time. Although the genes responsible for rapid lysis phenotype are located in three separate regions of the genetic map in T4, named *rI*, *rII* and *rIII*, only *rII* mutants are unable to grow on *E. coli* lysogenic for phage λ (Benzer 1955). The lambda genes responsible for *r* exclusion have been shown to be *rex* A and *rex* B (Matz et al 1982). These genes map in the immunity region of lambda and, along with *cI* repressor, are the only genes expressed by

lambda in the lysogenic state. It is Benzer's discovery of this non-permissive host that made the *rII* genes so valuable for genetic analyses. Using the complementation and recombination tests, Benzer found that all of the *rII* mutations are located in two cistrons with 47 small segments (Benzer 1959, 1961). Based on the response of *rII* mutations to specific bases, nucleosides or analogs and other chemical mutagens, Benzer and others identified indirectly numerous important *rII* mutations and gained insight into mechanisms of mutagenesis (Freese 1959, Champe and Benzer 1962, Drake 1970). Recently, both *rII* A *and rII* B genes (together about 3 kbp) have been sequenced (Pribnow et al 1981, Huang 1986, Daegelen and Brody 1990), which should facilitate the direct identification of the important genetic markers in the region, especially with the polymerase-chain-reaction (PCR) technique (Mullis and Falloona 1987, Erlich 1988, Gyllensten and Erlich 1988, Higuchi et al 1988, Saiki et al 1988).

While the *rII* genes are excellent markers for analysis of reversion mutations, their utility is limited by the lack of a simple forward mutation assay. In T4 bacteriophage, genes for lysozyme, thymidylate synthase, dihydrofolate reductase and thymidine kinase can potentially used as selectable markers (Chace and Hall 1973, Drake and Ripley 1983), in part because mutants in each gene can be identified by selective plating conditions.

9. Present work

In this thesis, I describe two projects which were done to further our understanding of mutagenic mechanisms associated with DNA precursor biosynthesis in vivo using the bacteriophage T4 system. The first is a study of the molecular basis of bromodeoxyuridine (BrdUrd) mutagenesis. As discussed earlier, BrdUrd was thought to induce dNTP pool imbalance, in which high BrdUTP pools could compete with depleted dCTP pools to base pair with guanine, leading to GC-to-AT transition in mammalian cells (Hopkins and Goodman 1980, Davidson et al 1988). Since dNTP pools are regulated through allosteric control of ribonucleotide reductase (Reichard 1985), the control of this enzyme should be a determinant of BrdUrd mutagenesis. Since T4 rNDP reductase is reported as a feedback-resistant enzyme, BrdUrd treatment was not expected to shrink hm-dCTP pool in T4 phage-infected cells; therefore, GC-to-AT transition was predicted to be dominant when phage rNDP reductase was replaced by the host counterpart, known to be a feedback-sensitive enzyme. Our preliminary results support our model, in that rNDP reductase is a determinant of BrdUrd mutagenesis (Sargent et al 1989). However, the mutation pattern is opposite to that which we would predicted. We found that AT-to-GC transition mutagenesis is stimulated more when *E. coli* rNDP reductase is active, while GC-to-AT transition mutagenesis is stimulated predominantly when the phage counterpart is active. To investigate the molecular basis for this observation, I analyzed the effects of BrdUrd on dNTP pools and mutagenesis. I also studied the response of rNDP

reductase to different deoxyribonucleoside triphosphates and found that under certain conditions CDP reduction is inhibited by BrdUTP or dTTP in T4 bacteriophage-infected cells.

Another project was designed to test the hypothesis that the mutator phenotype of a temperature-sensitive mutant with defective dCMP hydroxymethylase gene 42, *ts* LB3, is caused by localized dNTP pool perturbations. Early studies in Drake's and Greenberg's laboratories showed that certain *ts* gene 42 mutants at semipermissive temperature (34^{o} C), thought to partially inactivate dCMP hydroxymethylase, stimulate GC-to-AT transition mutations (William and Drake 1977). The original interpretation was that T4 dCMP hydroxymethylase may be required directly for DNA replication by interaction with proteins in the replication apparatus, such as DNA polymerase during DNA biosynthesis (Williams and Drake 1977, Chao et al 1977). However, in light of recent observations in this laboratory (Moen et al 1988, Thylén and Mathews 1989), one could visualize the *ts* gene 42 mutator phenotype as a consequence of depletion of hydroxymethyl-dCTP pool due to partial impairment of hydroxymethylase activity in vivo at semipermissive temperature, resulting in mutagenesis because of competition between correct and incorrect nucleotides at replication sites. To test this model, I constructed several *rII* x *ts* LB3 double mutants to quantitate mutation type and frequency induced by *ts* LB3 at semipermissive temperature. The dNTP pools were measured in phage-infected cell extracts. The status of replication-active dNTP pools was estimated based on direct DNA sequencing of mutations under mutagenic conditions. All the experimental results in this study are consistent with the hypothesis that the mutator phenotype of *ts* LB3 is a consequence

of perturbation of the flow of nucleotide precursors into the DNA replication machinery.

Lastly, I developed a fast forward mutation assay for investigating mutagenic specificity and DNA site specificity stimulated by imbalanced dNTP pools or other factors in vivo. Using the thymidine kinase gene as a marker, I further characterized the *ts* LB3 mutator phenotype by analyzing forward mutations stimulated by *ts* LB3 at a semi-permissive temperature (34^{o} C). Mutant genes were analyzed with an automatic DNA sequencer.

Table I-1. Genetic consequences of perturbation of dNTP pools[1]

Effect	Procaryotic cell	Eucaryotic cell
Mutation	+	+
Recombination	+	+
DNA breakage	+	+
Sensitization to mutagens	+	+
Chromosome/chromatin		
aberrations	NA[2]	+
breakage	+	+
Sister chromatid exchange	NA	+
Tumor promotion		
transformation	NA	+
Lethality	+	+

1 From de Serres (1985), Kunz (1988), Meuth (1989)

2 NA; not applicable

Table I-2. Comparison of allosteric enzymes in dNTP pool regulations in *E. coli* and T4 phage

E. coli	T4
A). rNDP reductase[1]	
CDP ↓dATP, dTTP, dGTP[2] / ↑ATP → dCDP	CDP ↑dATP, ATP, hm-dCTP → dCDP
UDP ↓dATP, dGTP, dTTP / ↑ATP → dUDP	UDP ↑dATP, ATP, hm-dCTP → dUDP
GDP ↓dATP, dGTP / dTTP → dGDP	GDP ↑dTTP → dGDP
ADP ↓dATP / ↑dGTP → dADP	ADP ↑dGTP → dADP
B). Thymidine kinase	
dThd ↓dTTP / ↑dCDP, dCTP, dADP → dTMP[3]	dThd ↓dTTP ? → dTMP[4]
C). dCTP deaminase[5]	dCMP deaminase[6]
dCTP ↓dTTP / ↑dCTP → dUTP	dCMP ↓dTTP / ↑hm-dCTP → dUMP

1 Larsson and Reichard (1966), Brown and Reichard (1969), Berglund (1972, 1975) and Thelander and Reichard (1979)
2 ↑ as activation and ↓ as inhibition
3 Iwatsuki and Okazaki (1967)
4 Allosteric property is unsolved. From Ritchie et al (1974) and Iwatsuki (1977)
5 Data are from *Salmonella typhimurium* (Beck et al 1975)
6 Data are from T2 dCMP deaminase. From Maley and Maley (1982) and Maley et al (1983)

Figure I-1. T4 genetic map

The numbers in the interior represent distance in kilobase pairs from a reference point, the *rIIA*/*rIIB* cistron divide. Although the T4 genome is a linear DNA molecule, the genetic map is circular due to circular permutation of base sequences in the genome (Mathews et al 1983)

Figure I-1

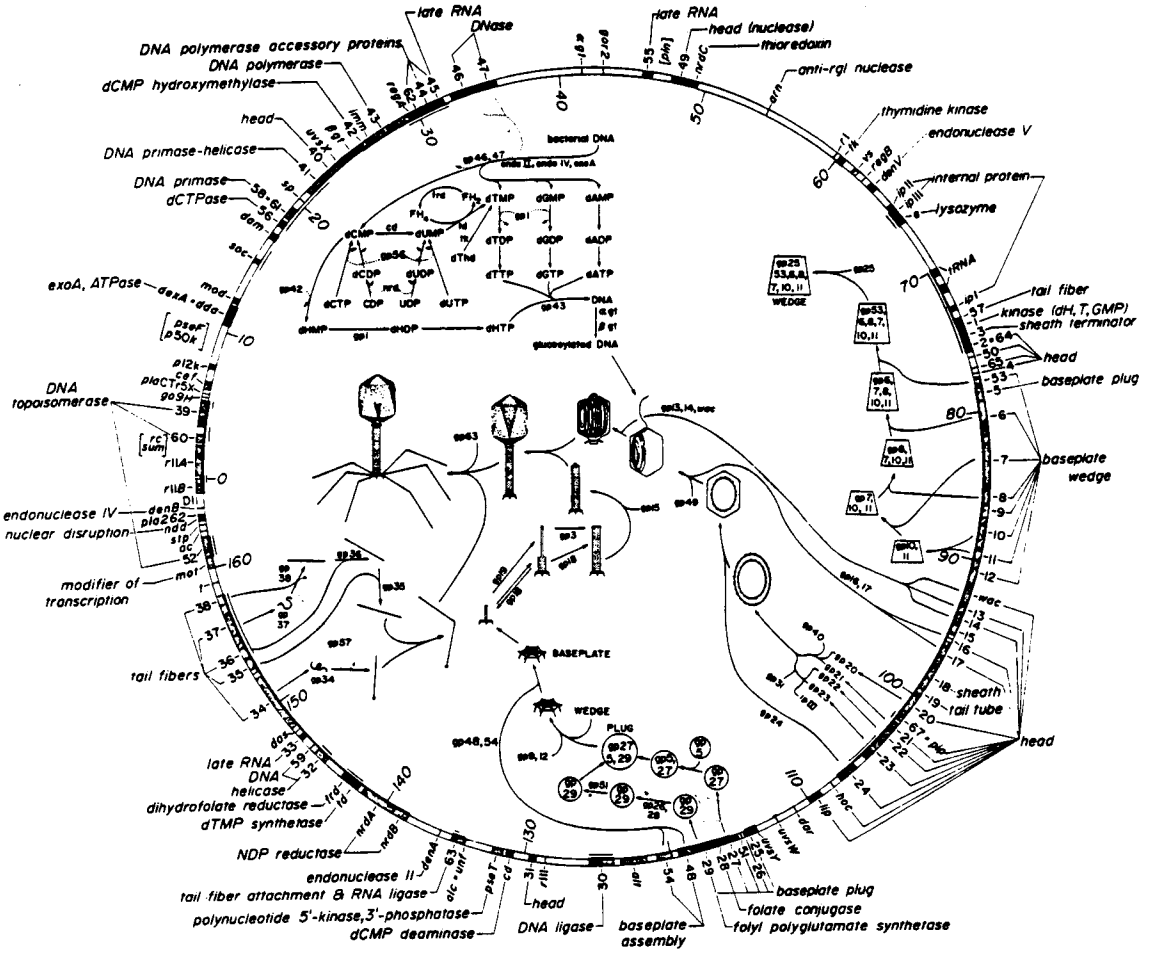

late RNA
DNase
DNA polymerase accessory proteins
DNA polymerase
dCMP hydroxymethylase
head
DNA primase-helicase
DNA primase
dCTPase
exoA, ATPase
DNA topoisomerase
endonuclease IV
nuclear disruption
modifier of transcription
tail fibers
late RNA
DNA helicase
dihydrofolate reductase
dTMP synthetase
NDP reductase
endonuclease II
tail fiber attachment & RNA ligase
polynucleotide 5'-kinase,3'-phosphatase
dCMP deaminase
DNA ligase
baseplate assembly
folate conjugase
folyl polyglutamate synthetase
baseplate plug
head
late RNA
head (nuclease)
thioredoxin
anti-rgl nuclease
thymidine kinase
endonuclease V
internal protein
lysozyme
tRNA
tail fiber
kinase (dH, T, GMP)
sheath terminator
head
baseplate plug
baseplate wedge
sheath
tail tube
head
bacterial DNA
glucosylated DNA
BASEPLATE
WEDGE
PLUG

Figure I-2. An overview of the T4 reproductive cycle

Immediately after injection of DNA into the host cell, early genes are transcribed, yielding enzymes involved in DNA precursor biosynthesis and DNA replication. Replication of the linear DNA molecule is initiated bidirectionally from multiple origins. Recombination among newly replicated molecules yields giant circular replicative intermediates. Replicating DNA sreves as the template for late gene transcription, yielding structural proteins. Independent subassembly pathways generate heads, tails, and tail fibers. Packaging of DNA into heads occurs concomitantly with head maturation and DNA replication. Newly formed virus particles are released by lysis of the cell (Mathews 1977).

Figure I-2

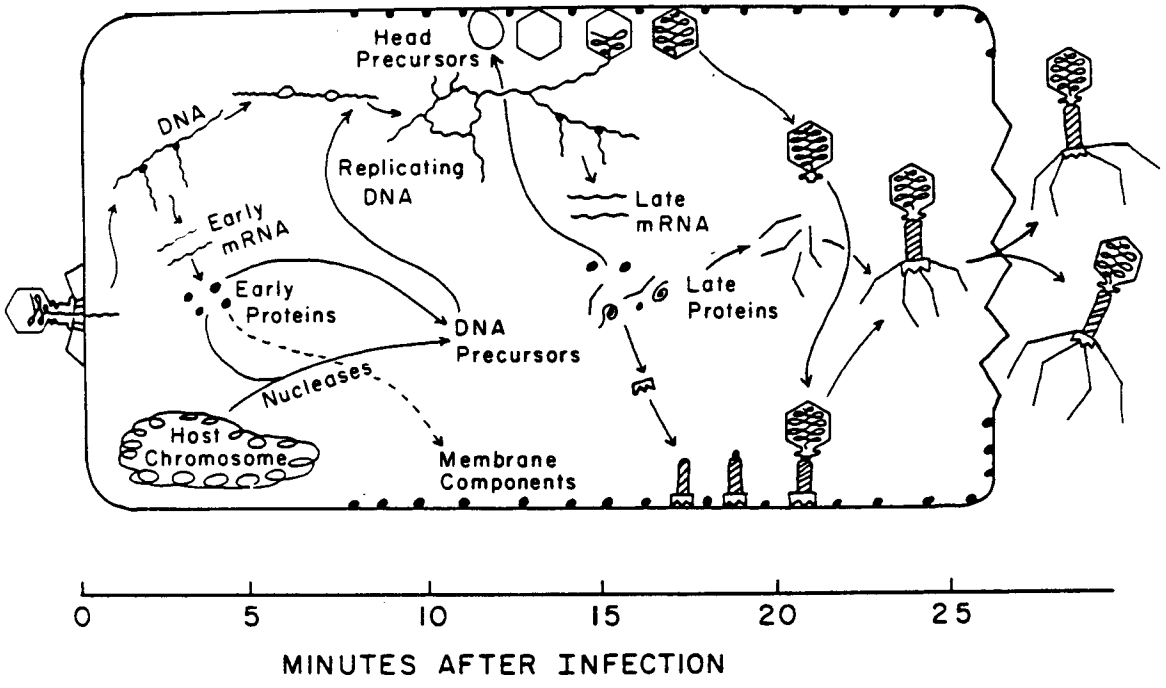

Head
Precursors
DNA
Replicating
DNA
Late
mRNA
Early
mRNA
Early
Proteins
Late
Proteins
DNA
Precursors
Nucleases
Host
Chromosome
Membrane
Components
0
5
10
15
20
25
MINUTES AFTER INFECTION

Figure I-3. Reactions of DNA precursor biosynthesis in T4 phage-infected *E. coli*

Reactions catalyzed by virus-coded and pre-existing host cell enzymes are denoted with heavy and light arrows, respectively (Mathews and Allen 1983)

Figure I-3

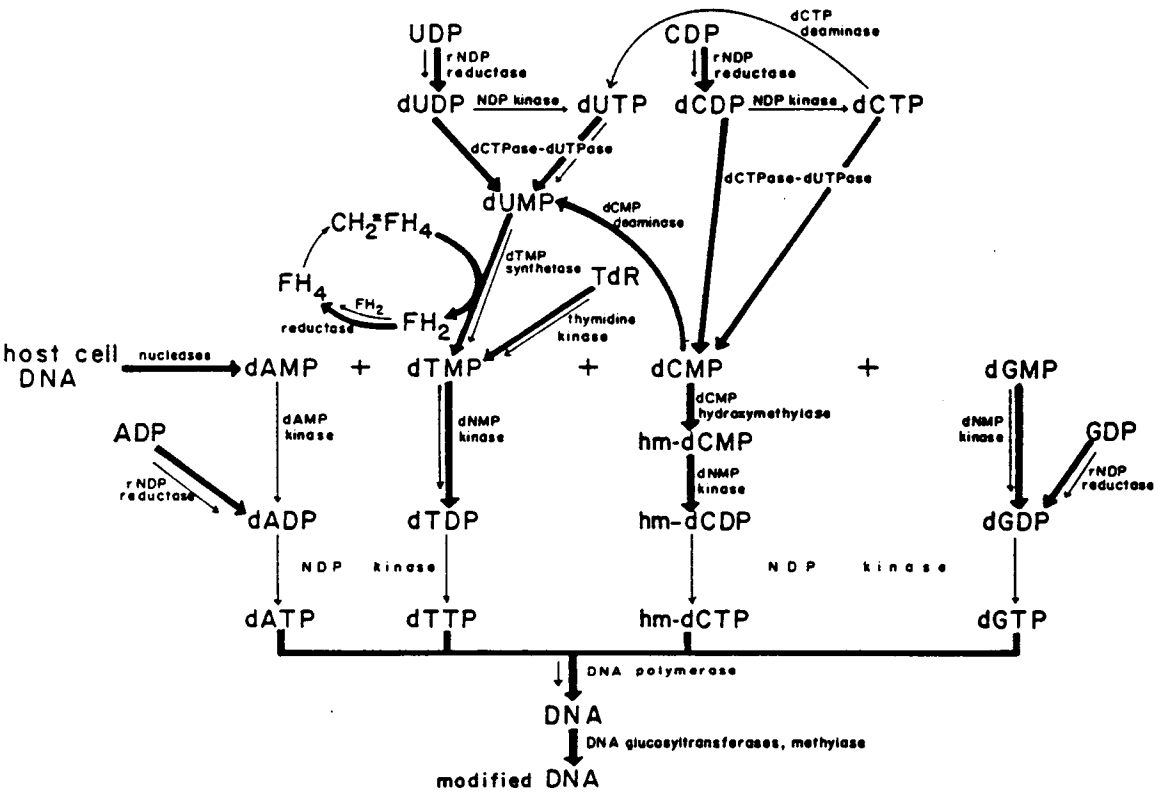

UDP
rNDP reductase
dUDP
NDP kinase
dUTP
CDP
rNDP reductase
dCDP
NDP kinase
dCTP
dCTP deaminase
dCTPase-dUTPase
dCTPase-dUTPase
dUMP
dCMP deaminase
$CH_2{=}FH_4$
dTMP synthetase
TdR
FH_4
FH_2
reductase
FH_2
thymidine kinase
host cell DNA
nucleases
dAMP
+
dTMP
+
dCMP
+
dGMP
dCMP hydroxymethylase
dAMP kinase
dNMP kinase
dNMP kinase
ADP
GDP
rNDP reductase
rNDP reductase
hm-dCMP
dNMP kinase
dADP
dTDP
hm-dCDP
dGDP
NDP kinase
NDP kinase
dATP
dTTP
hm-dCTP
dGTP
DNA polymerase
DNA
DNA glucosyltransferases, methylase
modified DNA

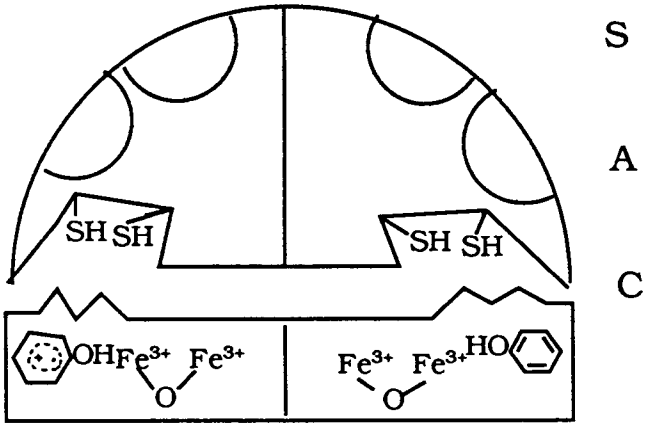
Figure I-4. Schematic model of ribonucleotide reductase from *E. coli*

A. Model for structure. The protein is a heterotetramer with two large subunits and two small subunits. Three functional sites are identified as substrate specificity site (S), activity site (A) and catalytic site (C).

B. Model for allosteric regulation. Feedback inhibition is denoted by solid lines ending in open rectangular boxes and allosteric activation is denoted by dashed lines (Thelander and Reichard 1979).

Figure I-4

A


S
A
C
SH SH
SH SH
OH
Fe3+ Fe3+
O
Fe3+ Fe3+
HO
O

B

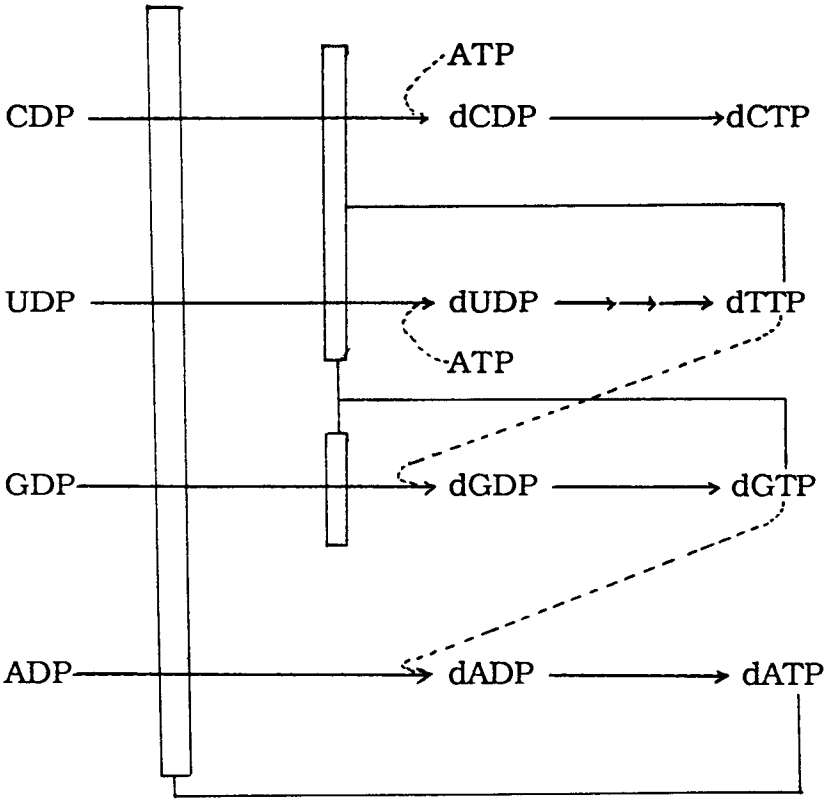
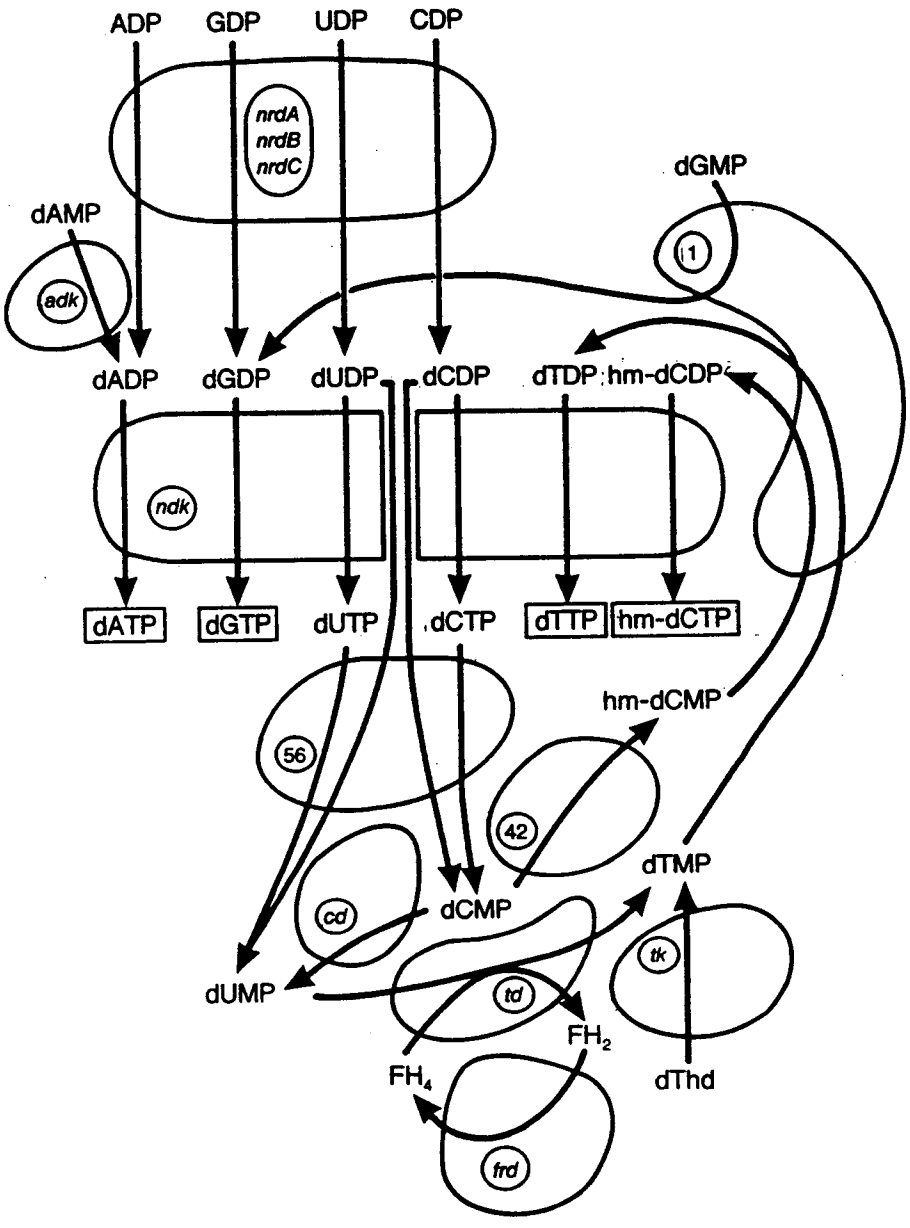

ATP
CDP
dCDP
dCTP
UDP
dUDP
dTTP
ATP
GDP
dGDP
dGTP
ADP
dADP
dATP

Figure I-5. Enzyme activities detected in the T4 dNTP-biosynthesizing multienzyme complex

The structural gene for each enzyme is shown: *nrd* A/B/C, rNDP reductase and thioredoxin; *adk*, dAMP kinase; *cd*, dCMP deaminase; *ndk*, NDP kinase; *frd*, dihydrofolate reductase; *td*, thymidylate synthase; *tk*, thymidine kinase; *1*, dGMP kinase; *42*, dCMP hydroxymethylase; *56*, dCTPase-dUTPase. Note that dAMP kinase and NDP kinase are bacterial gene products (Mathews et al 1988)

Figure I-5


ADP
GDP
UDP
CDP
nrdA
nrdB
nrdC
dGMP
dAMP
1
adk
dADP
dGDP
dUDP
dCDP
dTDP
hm-dCDP
ndk
dATP
dGTP
dUTP
dCTP
dTTP
hm-dCTP
hm-dCMP
56
42
dTMP
cd
dCMP
tk
dUMP
td
FH2
FH4
dThd
frd

Chapter II

Analysis of Bromodeoxyuridine Mutagenesis Reveals Allosteric Control of T4 Phage Ribonucleotide Reductase

Running title: T4 rNDP reductase and BrdUrd mutagenesis

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1. Abstract

Further study of bromodeoxyuridine mutagenesis using a "metabolic engineering experiment" with a large subunit-defective T4 mutant, in which phage used *E. coli* rNDP reductase for DNA precursor biosynthesis, confirmed our previous hypothesis that rNDP reductase is a determinant of bromodeoxyuridine mutagenesis in T4 bacteriophage. GC-to-AT transitions were stimulated predominantly when T4 rNDP reductase was active. Direct sequencing of *rII* reversion mutations stimulated by bromodeoxyuridine suggests that the significant bias of deoxyribonucleoside triphosphate (dNTP) pool ratio around replication sites is that of bromo-deoxyuridine triphosphate pool (Br-dUTP) to hydroxymethyl-deoxycytidine triphosphate pool (hm-dCTP). By directly examining the control by deoxynucleoside triphosphates on reduction of cytidine diphosphate (CDP) reduction in vitro, we found that under certain conditions CDP reduction was substantially inhibited by Br-dUTP or dTTP in T4 bacteriophage.

Key words: BrdUrd mutagenesis; replication fidelity; dNTP pools; T4 rNDP reductase; T4 dNTP synthetase multienzyme complex

2. Introduction

Bromodeoxyuridine (BrdUrd) can induce both incorporation errors and replication errors through mispairing of bromouracil with guanine during DNA replication (Freese 1959, Drake 1970). The mutagenic pathways stimulated by BrdUrd, including GC-to-AT and AT-to-GC transitions as shown in Figure II-1, should be sensitive to dNTP pool fluctuations. Since dNTP pools are regulated in large part through allosteric control of rNDP reductase, the control of this enzyme could be a determinant of BrdUrd mutagenesis (Hopkins and Goodman 1980). Studies in mammalian systems showed that the dCTP pool is depleted in cells cultured in BrdUrd-containing medium (Ashman and Davidson 1981), while the principal mutagenic pathway stimulated by BrdUrd is GC-to-AT transition (Davidson et al 1988). These results support a model in which the mutagenic effect of BrdUrd is based on dNTP pool perturbation. According to this model, BrdUTP functions as an analog of dTTP, known to be an allosteric inhibitor for CDP reduction, leading to dCTP pool depletion, thereby favoring the competition between BrdUTP and dCTP to mispairing with guanine in mammalian cell DNA. However, in studies of T4 bacteriophage-infected *Escherichia coli*, in which viral rNDP reductase is reported to be feedback-resistant (Berglund 1972, see Chapter I-3 & 9), predominant mutations of GC-to-AT were also observed. Furthermore, no significant perturbations of dNTP pools were detectable in T4-infected cells following BrdUrd treatment (Sargent et al 1989). These observations suggested that the effect of BrdUrd on T4 DNA precursor metabolism is more complex than anticipated.

The observation of normal dNTP pool sizes in crude cell extracts

under BrdUrd mutagenesis condition does not rule out the possibility of local perturbation of dNTP pools around replication forks. Several studies suggest that there exist two functionally differentiated dNTP pools in procaryotic cells (Reddy and Mathews 1978, Manwaring and Fuchs 1979, Mathews and Sinha 1982, Ji and Mathews 1991). One small, rapidly replenished pool is proposed to be located close to replication sites, directly supplying dNTPs for replication, while another much larger, more highly dispersed pool is more remote from replication sites, and can be used for repair and regulation of dNTP synthesis. Since there is no direct approach to detect such replication-active dNTP pools, the study of specific gene sequence alterations could yield some insight about dNTP pools around replication sites.

The goal of this study is to further test our model that rNDP reductase is a determinant of BrdUrd mutagenesis in T4-infected cells. We switched the source of large subunits of rNDP reductase from either host or virus for phage DNA precursor biosynthesis, concluding that rNDP reductase is a determinant for BrdUrd mutagenesis. By examining the allosteric properties of rNDP reductase, we also found that phage CDP reductase activity is substantially inhibited by either BrdUTP or dTTP.

3. Materials and methods

Reagents

Radioactively labeled [^{3}H] deoxyribonucleoside 5'-triphosphates, [8-^{3}H]dATP, [methyl-^{3}H]dTTP, [5-^{3}H]dCTP and [8-^{3}H]dGTP were purchased from ICN Pharmaceuticals. [5-^{3}H] cytidine 5'-diphosphate and [γ-^{32}P] rATP were obtained from New England Nuclear. Deoxyribonucleoside triphosphates, dideoxyribonucleoside triphosphates and copolymers poly (dA-dT) and poly (dI-dC) were from Pharmacia. Bromodoxyuridine was from Calbiochem. Other ribonucleosides, deoxyribonucleosides, ribonucleotides and deoxyribonucleotides were from Sigma. Phenylmethylsulfonyl fluoride (PMSF) was from Boehringer-Mannheim. Tri-N-octylamine was from ICN Pharmaceuticals, and Freon-113 (1,1,2-trichlorotrifluoroethane) was from Aldrich Chemical Company.

Taq DNA polymerase used for polymerase-chain-reaction was from Promega. Purified *E. coli* DNA polymerase I used for dNTP pool assay was from Boehringer-Mannheim. Polynucleotide kinase used for end-labelling of nucleotides was from New England Biolabs. DNA sequenase (version 2.0) was from USB. DNAase I and RNAase were from Sigma.

Primers used for polymerase-chain-reaction and DNA sequencing were synthesized on a model 380B DNA synthesizer from Applied Biosystems Inc. (ABI) by Dr. R. McParland of the Center for Gene Research and Biotechnology, Oregon State University.

Media

Nutrient broth contained 8 g of Difco nutrient broth and 5 g NaCl per liter; nutrient agar plates contained 4 g nutrient broth, 5 g NaCl and 23 g of Difco nutrient agar per liter. M-9 medium contained 1 g NH_4Cl, 6 g Na_2HPO_4, 1 g NaCl, 0.01 g $CaCl_2$, 0.2 g $MgSO_4$-$7H_2O$ and 3 g glucose. SM9 medium is M9 with addition of 2 g vitamin-free casamino acids per liter.

Bacterial and phage strains

E. coli strains, B, BB and K38(λ) and T4 phage strains, T4D, *nrd am* A67, *rII* UV215 and *rII* SN103 were from this laboratory. *E. coli* ED8689, a sup^o, $hsdR^-$, $hsdM^+$, K12 strain (Wilson et al 1977) was transformed either with pBR322 or with pPS2, a pBR322-derived plasmid containing the *nrd* A and *nrd* B genes of *E. coli* (Platz and Sjöberg 1980, Sargent et al 1989). Strains carrying pPS2 express both subunits of *E. coli* rNDP reductase at levels about ten-fold higher than normal (Platz and Sjöberg 1980). The genotypes or phenotypes of strains used in this study are listed in Table II-1.

Phage crosses

Crosses between *rII* mutants, *rII* UV215 or *rII* SN103 and ribonucleotide reductase amber mutant, *nrd am* A67 were performed as described by Hall et al (1967). A mixture of *nrd* mutant and *rII* mutant phages at 10 : 1 ratio was added to 1 ml fresh *E. coli* BB (2 X 10^8 cell/ml) at a multiplicity of 6 of nrd^+ and *rII* phage together. The

infected cultures were incubated one hour at 37^{o} C for complete lysis. These lysates were diluted and plated on *E. coli* B or BB. The phage plaques were picked and plated on *E. coli* K38(λ). The phage which could not plate *E. coli* K38(λ) were collected as *rII*$^{-}$ phage (Benzer 1961). Further screening of each *rII*$^{-}$ and *nrd am* A67 double mutant was based on the ribonucleotide reductase activity assay (Slabaugh et al 1984).

Preparation of cell cultures for enzyme assay

A culture of *E. coli* B was grown at 37^{o} C, with aeration, to a cell density of 2.5 x10^{8} cells per ml in nutrient broth or SM9 medium. After addition of L-tryptophan to 20 μg/ml, the cells were infected with phage immediately at a multiplicity of 6 phages per bacterium. The cells were harvested after infection for 16 minutes by rapidly chilling the cells on ice and then centrifuging at 6000 X g for 10 minutes. The cell pellets were resuspended in 50 mM Tris buffer, pH 7.5, containing 4 mM dithiothreitol, 10 mM magnesium acetate and 0.2 mM PMSF. The cells were disrupted gently by four 10-second bursts of sonic oscillation with intermittent cooling periods. The homogenate was centrifuged at 12,000 X g for 15 minutes. The supernatant was used directly as enzyme source for rNDP reductase assay. In some experiments, noted in the text, the crude extract was further fractionated by 1% streptomycin sulfate precipitation and 35% ammonium sulfate precipitation as described by Slabaugh and Mathews (1986). This enzyme preparation is referred to as partially purified enzyme. Further purification was conducted by Fast-Protein-Liquid-Chromatography (FPLC) with a Superose-6 column. All the enzyme preparation steps were carried out at 4^{o} C.

Ribonucleotide reductase assay

The ribonucleotide reductase activities were measured essentially as reported by Slabaugh et al (1984) with minor modifications. The 40-µl standard reaction mixture contained 100 µM (4-2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), pH 8.0, 10 mM dithiothreitol, 20 µM $FeCl_3$, 4 mM AMP-PNP, 2 mM magnesium acetate, 50 µM [^{3}H]-CDP (50 cpm/pmol) and enzyme extract. After incubation at 37^0 C for 10 and 20 minutes, reactions were terminated by adding 4.4 µl of 10 M perchloric acid, and then the reaction mixtures were stored on ice. The supernatant after centrifugation was heated to 100^0 C for 30 minutes to hydrolyze nucleoside polyphosphates to nucleoside monophosphates. Samples were cooled on ice and 4 µl of marker solution containing CMP, dCMP and dUMP each at 20 mM was added. The acidic solution was neutralized with 5 M KOH and the potassium perchlorate precipitate was removed by centrifugation. 20-µl aliquots of each supernatant were spotted on cellulose plastic-backed thin-layer chromatographic plates. Chromatograms were developed overnight with a solvent composed of ethanol-saturated sodium tetraborate-5 M ammonium acetate (pH 9.8)-250 mM EDTA (220:80:20:1, v/v/v/v). The spots containing dCMP-dUMP were identified under ultraviolet light and cut out from the plate for counting radioactivity. All assays were carried out in triplicate, and identical assays agreed within 10%.

rII reversion assay

Measurement of mutation rates in T4-infected *E. coli* were done essentially as described by Drake (1970). Fresh *E. coli* BB cells were grown to a density of 2.5 X 10^8 cells per ml and diluted to a density of 2

X 10^7 cells per ml. 10 to 100 phages were added to a 5-ml diluted cell culture and the infections were stopped by addition of a few drops of chloroform after incubation of culture at 37^o C for 4 hours with aeration. Phage in the lysates were plated on nutrient broth plates containing either *E. coli* BB or *E. coli* K38(λ) for determining the revertant fraction.

dNTP pool determinations

25-ml *E. coli* strains were grown at 37^o C to a density of 2 X 10^8 cells/ml in SM9 medium. After addition of L-tryptophan to 20 μg/ml, the cells were infected with phage at a multiplicity of six phages per bacterium. Uninfected cultures were treated identically except that no phage was added. The cultures were harvested by rapid filtration after infection for 16 minutes at 37^o C. Deoxyribonucleoside 5'-triphosphate pools were extracted with 5% trichloroacetic acid twice and neutralized with 0.5 M tri-N-octylamine/Freon, as described by Sargent and Mathews (1987). The principle in the dNTP pool assay was based on incorporation of a limiting dNTP in a cell extract into an alternating copolymer template [poly (dA-dT) or poly (dI-dC)] by DNA polymerase I in the presence of an excess of the labeled complementary dNTP. The experimental procedures were reported by Sargent and Mathews (1987) with modifications as follows. 100 μM dAMP, instead of 10 mM, was added to each dNTP pool assay reaction mixture to prevent template breakdown due to the 3'-to-5' exonuclease activity of *E. coli* DNA polymerase. Each dNTP pool assay was repeated twice, and variations between replicate assays were less than 10%. The sizes of dNTP pools are expressed as number of molecules of dNTP per cell.

Purification of T4 phage DNA

The T4-infected crude cell lysate was incubated with ribonuclease A and DNAase I (10 μg/ml each) at 37° C for 30 minutes and centrifuged at 5,000 X g for 15 minutes. The phage particles in each supernatant were concentrated by centrifugation at 35,000 X g for 30 minutes. Alternatively, phage particles were precipitated by centrifugation at 10,000 X g for 20 minutes after addition of polyethylene glycol 6000 to 20% (w/v) and NaCl to 2.5 M final concentration. The phage pellet was resuspended in one fifteenth the original volume in TE buffer containing 10 mM Tris-1 mM EDTA, pH 8.0. The phage DNA was further purified by phenol and chloroform extraction followed by ethanol precipitation (Maniatis et al 1982).

Primer purification

The primers after synthese in the DNA synthesizer were purified by 20% polyacrylamide gel electrophoresis. The desired oligonucleotide band shown in the gel under ultraviolet light with fluorescent PEI cellulose plate on the back was cut into small pieces and incubated with Sep-Pak buffer containing 0.1 M Tris (pH 8.0), 0.5 M NaCl and 5 mM EDTA at 37 °C overnight. The primers were further purified with C_{18} Sep-Pak cartridges. Salts were eluted from the crude mixture by adding 20 ml deionized H_2O after primers were loaded into each cartridge. The desalted primers were eluted with 3 ml of a mixture of 50 mM triethylammonium acetate and methanol (1 : 1 v/v) and evaporated to dryness. The purified primers were dissolved in TE buffer at 50 pmoles/μl and stored at -20° C for PCR and DNA sequencing (Maniatis et al 1982).

Amplification of genomic DNA

Phage DNA was subjected to amplification with polymerase-chain-reaction as described (Mullis and Faloona 1987, Saiki et al 1988) with modifications. Each reaction mixture contained 10 mM Tris (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$ and 25 μM of each dNTP (dATP, dTTP, dCTP, dGTP), two primers at 50 pmoles each and 2 units of *Taq DNA* polymerase with 10 pg of phage DNA as template. The 100-μl reaction mixture was overlaid with 50 μl of mineral oil to prevent evaporation and subjected to 35 cycles of amplification as follows. The samples were heated at 93^0 C for 1 minute to denature the DNA, cooled at 40^0 C for 2 minutes to anneal the primers and heated to 73^0 C for 2 minutes to amplify the template. Each additional cycle was run at 93^0 C for 1 minute, 40^0 C for 2 minutes and 72^0 C for 10 minutes to ensure that the final extension step was complete. All the thermal cycling was performed in a programmable heat block thermal cycler from Ericcomp Inc. The PCR products were identified on a gel of 1.5% regular agarose or 1% regular agarose with 3% NeuSieve agarose in TBE buffer (89 mM Tris - 89 mM borate - 2 mM EDTA) and stained with ethidium bromide (Maniatis et al 1982).

PCR product purification

Three methods were used for purification of PCR products after template amplification. 1) Centricon-30 or -100 devices were used to remove primers and deoxyribonucleotides as follows. Each PCR mixture was transferred to a Centricon reservoir, diluted with 1 ml of deionized H_2O and centrifuged for 30 minutes at 5000 X g (Centricon-30) or 1000 X g (Centricon-100) in a centrifuge with a fixed angle rotor. This

dilution and centrifugation step was repeated twice. About 40 µl of final concentrate was recovered. 2) Polyethylene glycol 6000 (PEG 6000) was used to remove primers and nonspecific amplified short oligonucleotides. Each PCR mixture was incubated with 0.6 volume of PEG-NaCl (20% W/V PEG 6000 and 2.5 M NaCl) for 10 minutes at 37^{o} C. PCR products were precipitated by centrifugation for 10 minutes at 16,000 X g. The pellets were washed once with 80% ethanol, dried with vacuum and dissolved in TE buffer. 3). The desired oligonucleotide band shown in the 1.5% agarose gel stained with ethidium bromide under ultraviolet light was cut out. The DNA was extracted with phenol/chloroform and precipated with ethanol (Maniatis et al 1982). Satisfactory results were obtained from all three methods.

DNA sequencing

The amplified templates after purification with Centricon-30 were sequenced by the chain termination method (Sanger et al 1977), using DNA sequenase as reported by Higuchi et al (1988) with some modifications. Oligonucleotide primers were 5' end-labeled with [γ-^{32}P]ATP and polynucleotide kinase. The end-label reaction mixture contained 10 units of polynucleotide kinase, 10 pmoles of primer, 10 pmoles of [γ-^{32}P]ATP, 0.01 M $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.01 mM EDTA, and 0.05 M Tris-HCl, pH 7.6. The reaction mixture was incubated at 37^{o} C for 45 minutes to end-label primer, then at 90^{o} C for 2 minutes to terminate the reaction. The labeled primer was mixed with PCR-amplified DNA template and heated at 90^{o} C for 5 minutes to denature DNA, then immediately stored on ice to anneal template with primer. Each sequencing reaction was initiated by

adding 2.8 µl of this mixture to 3.25 µl of dideoxy A, T, G, and C reaction mixtures composed of reagents provided in the "sequence kit" as follows: 2.5 µl of the A, T, G and C "termination mixtures", 0.15 µl of sequenase (1 unit), 0.38 µl of "5X buffer", and 0.22 µl of 0.1 M DTT. After a six-minute incubation at 37^{o} C, each reaction was stopped by adding 4 µl of 95% (v/v) formamide/20 mM EDTA; next, each reaction mixture was heated to 85^{o} C for 2 minutes, and loaded into a 7% polyacrylamide/7M urea gel. Electrophoresis was carried out at 40 watts, followed by drying and exposing of gel to Kodak X-Omat film.

4. Results

Rationale for this study

If BrdUrd mutagenizes in part through inhibition of CDP reduction by BrdUTP, then this effect would be most pronounced under conditions where a feedback-sensitive form of rNDP reductase is functioning (see Chapter I-9). Since this effect would enhance the ability of BrdUTP to compete with a mixture of 5-hydroxymethyldeoxycytide triphosphate (hm-dCTP) and dCTP for incorporation opposite a template guanine, BrdUrd should stimulate mutagenesis under these conditions primarily along a GC-to-AT transition pathway. Therefore, if we switched T4 rNDP reductase, reported to be a feedback-resistant enzyme, with its host counterpart, a feedback-sensitive enzyme, we would expect to reverse the mutation pathways. The functioning of the *E. coli* enzyme should correlate with primary stimulation of GC-to-AT transition mutagenesis, while functioning of the feedback-resistant T4 enzyme should correlate with stimulation of an AT-to-GC pathway.

Making T4 DNA synthesis dependent upon *E. coli* rNDP reductase

It is possible to make T4-infected cells wholly dependent upon *E. coli* rNDP reductase for dNTP synthesis (a "reductase swich" experiment or "metabolic engineering" experiment), by directly infecting *E. coli* host with a T4 rNDP reductase-defective mutant. However, since the activity of the phage enzyme is about ten-fold higher than that of the host counterpart enzyme, direct infection of *E. coli*

wild strain with a rNDP reductase-defective mutant could affect mutagenesis by limiting the supply of deoxyribonucleotides. If the rate of DNA replication were limited by dNTP availability, the newly replicated DNA would be proofread more efficiently (Sinha and Goodman 1983), and mutagenesis rates would vary independently of the presence or absence of BrdUrd. Therefore, we used a host *E. coli* ED8689/pPS2, which bears a multi-copy plasmid overproducing *E. coli* rNDP reductase by about ten-fold. To confirm that the supply of dNTPs for phage DNA replication in the phage-infected over-producing host was not limiting, we determined the phage yield for different phage-host combinations, as shown in Table II-2. Infection of the over-producing host by rNDP-deficient phage (T4 *nrd am* A67) yield nearly as high a burst size as does wild type T4, while the burst size in the non-overproducing host (ED8689/pBR322) with infection of *nrd am* A67 was reduced 80%. This suggests that phage DNA replication is essentially independent of limitation by the rate of DNA synthesis during infection of T4 *nrd* mutants in our experiments.

5-Bromodeoxyuridine mutagenesis

By using different *rII* mutations, which should revert to wild type by either AT-to-GC or GC-to-AT transitions, we found that both kinds of mutations were stimulated by bromodeoxyuridine, as shown in Table II-3. The pattern of stimulation of mutation depends on the source of rNDP reductase. The GC-to-AT transition was enhanced more when the T4 enzyme was active. The AT-to-GC transition was stimulated predominantly when the host rNDP reductase was functionning. Meanwhile, spontaneous reversion mutations by both pathways were

also stimulated about 2- to 4-fold when the T4 rNDP reductase was replaced by host protein. These results are consistent with early studies in this laboratory (Sargent 1987), supporting our hypothesis that rNDP reductase is a determinant for BrdUrd mutagenesis, and that the integrity of the T4 dNTP-synthesizing multienzyme complex contributes to replication fidelity.

DNA sequence

Based on our model, GC-to-AT transition mutagenesis should be minimal when T4 rNDP reductase is active. However, our observations contradicted this prediction. To confirm the apparent reversion pattern enhanced by T4 rNDP reductase under BrdUrd treatment, we directly sequenced the genetic marker, *rII* SN103. This *rII* mutation was determined previously based on response of mutation to chemical mutagens (Benzer 1961, Drake 1970). Based on the *rII* genetic map (Benzer 1961) and partial *rII* DNA sequence (Pribnow et al 1981), we designed two oligonucleotide primers and sequenced the *rII* SN103 mutation after amplification of the template by PCR. As shown in Figure II-2, a 200-base pair template was amplified as expected. The *rII* SN103 mutation was identified after direct sequencing of the PCR-amplified template, located at base-pair #265 from the *rII* B translation start with an T-to-C transition, as shown in Table II-4. Nine revertants stimulated by BrdUrd under the function of T4 rNDP reductase were sequenced; 8 were GC-to-AT transitions at the mutant site or its immediate 3' neighboring cytosine, as shown in Table II-5. We propose that these transition mutations occur through a guanine-bromouracil (G-BU) mismatch intermediate, resulting from competition of BrdUrd with hm-dCTP and incorporation opposite template guanine, as shown in

Table II-6. These results suggest that the mutagenic basis of BrdUrd is expansion of the [BrdUTP + dTTP] and depletion of the hm-dCTP pool, leading to a GC-to-AT transition as a predominant mutation pathway in T4 *nrd*$^+$ phage-infected cells.

dNTP pools

Do such disturbed dNTP pools apparently induced by BrdUrd exist throughout the whole cell? In other words, could such biased dNTP pool be detectable with our established dNTP pool assay? As shown in Figure II-3 and Table II-7 there was less than 10% fluctuation of dNTP levels among all treatments, no matter which rNDP reductase was active and whether or not BrdUrd was added. These results are reminiscent of observations made earlier with an T4 *nrd am* B mutant, defective in synthesis of the small subunit of NDP reductase (Sargent et al 1989). In both cases, experimental data suggest that perturbations of dNTP pools under BrdUrd treatment are undetectable in cell extracts.

Expansion of dNTP pools, especially the dGTP pool, was observed when the *E coli* rNDP reductase-overproducing host was infected with a small subunit-defective T4 mutant (Sargent et al 1989). However, no such severe expansion of dNTP pools was observed when the same host was infected with a T4 large subunit-defective phage. The patterns of dNTP pool changes after infection with T4 *nrd am* A67 were more like those seen in infection with wild type T4 than with of *nrd am* B55 (Sargent et al 1989).

Allosteric effects of BrdUTP on rNDP reductase

The BrdUrd mutagenesis when T4 rNDP reductase was active suggested that the CDP reductase activity of this enzyme was sensitive to allosteric inhibition, in contrast to an earlier report by Berglund (1972). Therefore, we directly examined the allosteric effects of dNTPs on CDP reduction upon T4 rNDP reductase. We found that the enzyme in crude extracts was sensitive to allosteric moderators, as shown in Figure II-4. Both dTTP and BrdUTP inhibited CDP reduction, while dATP stimulated activity. The allosteric inhibition was observed at concentrations of pyrimidine dNTPs as low as 0.01 mM. In order to further study this allosteric behavior, we purified T4 rNDP reductase by ammonium sulfate fractionation, followed by FPLC separation on a Superose-6 column. The purified protein was identified by SDS-PAGE and Western blotting. However, we found the purified form of the enzyme was insensitive to either BrdUTP or dTTP (data were not shown). In fact, ammonium sulfate fractionation alone is sufficient to abolish the allosteric inhibition. As shown in Figure II-5, we found that the partially purified T4 NDP reductase, with CDP as substrate, was totally insensitive to inhibition by 1 mM dTTP or 5-BrdUTP, while it was stimulated by dATP. The allosteric property of partially purified enzyme was in good agreement with Berglund's observation (Berglund 1972). As one control in our experiments, we also studied the allosteric behavior of the *E. coli* rNDP reductase allosteric property. We found that crude or partial purified preparations of the enzyme from uninfected *E. coli* responded identically to allosteric mediators (Figure II-6). These observations indicate that BrdUTP and dTTP could function as allosteric inhibitors for CDP reduction, at least in crude extracts.

5. Discussion

An "metabolic engineered" T4 dNTP synthetase complex increases spontanous mutations

Studies have shown that T4 DNA precursor-biosynthesizing enzymes associate together by protein-protein interactions, and form a multienzyme complex in vivo (Mathews et al 1988). More than ten gene products have been identified in this T4 dNTP synthetase complex (Moen et al 1988). The physiological significance of the existence of such dNTP multi-enzyme complexes is thought to involve efficiency and control of cellular processes during DNA replication. By using two criteria for an intact complex -- kinetic coupling among constituent enzymes in crude extracts of infected bacteria, and co-elution of enzyme activities from a gel filtration column -- Moen et al (1988) found that both subunits of T4 rNDP reductase were essential for the formation of an intact complex. However, the large subunit of phage rNDP reductase could apparently be replaced by a host protein to form an intact complex, while the small subunit of enzyme could not be replaced by a host protein. Does such a "dNTP synthetase" complex with replacement by a host protein function like the native complex for coordinating replication processes in vivo? By studying phage yields and spontaneous mutation frequencies, we found here that replacement of the large subunit of T4 rNDP reductase with host protein could restore the phage burst size essentially to that seen with wild-type phage. However, the spontaneous mutation rate was enhanced, by 2- to 4-fold in both AT-to-GC and GC-to-AT transition pathways. The difference between T4 and host large subunit is more functional than structural (see Chapter I-3), suggesting that coordinating the reaction of individual proteins due to

their distinct kinetic or allosteric property in this complex contributes toof large subunit the fidelity of DNA replication.

Is T4 rNDP reductase a feedback-sensitive enzyme in vivo?

The observation of different responses to allosteric mediators of T4 rNDP reductase, in terms of CDP reduction, in two different enzyme preparations suggested that the regulation of T4 rNDP reductase is more complicated than what Berglund (1972) observed in purified enzyme in vitro. It appears that we can view such complexity of regulation in the light of protein-protein interactions in the dNTP synthetase complex. A crude extract of gently lysed cells, which should contain rNDP reductase as part of the dNTP multienzyme complex, showed strong inhibition of CDP reduction by both BrdUTP and dTTP, while an ammonium sulfate fraction, in which ionic contacts would have disrupted the protein-protein interactions, showed no inhibition of CDP reduction by pyrimidine nucleoside triphosphates.

These results suggest that the regulatory behavior of this key enzyme is modified by interactions of rNDP reductase with other proteins, possibly by its assembly into T4 dNTP synthestase multienzyme complex. The experimental data in vivo showed this could be the case. By studying mutagenic effects of a T4 dCMP deaminase-defective mutant, Sargent (1987) found the hm-dCTP pool in phage-infected cells was sensitive to an exogenous supply of thymidine. More than 50% of hm-dCTP pool was depleted when T4 infected-cells were grown in 50 μM thymidine-containing medium, suggesting that cytidylate reduction by rNDP reductase is inhibited by high dTTP pools in vivo. However, our attempts to reconstitute a feedback-sensitive form of T4 rNDP reductase in vitro have not succeeded so far.

Are dNTP pools imbalanced around replication forks?

One interesting observation in this experiment is that no perturbation of dNTP pools was detectable after BrdUrd treatment when either viral or host rNDP reductase was active. In fact, we did not observe BrdUTP and dTTP pool accumulation even after treatment with 100 μM BrdUrd. Since the kinases which would convert BrdUrd to BrdUTP are present in ample amounts in T4-infected bacteria (Mathews and Allen 1983), the failure of the dTTP analog, BrdUTP, to accumulate suggests that the dTTP pool size is regulated by an uncharacterized mechanism. Consistent with this, Sargent (1987) found that overproduction of thymidylate synthase in *E. coli* by as much as 30-fold, does not significantly expand the dTTP pool.

However, the dNTP pools measured here tell us only about total amount of dNTPs per cell, but not about dNTP concentrations around replication forks. It is clear that the dNTP pools around replication forks are more crucial than total amount of dNTPs inside cells in determining the accuracy of DNA replication. Several lines of evidence here suggest that BrdUTP and dTTP pools are expanded, while the hm-dCTP pool is depleted around replication forks. First, high mutation rates stimulated by BrdUrd strongly suggest that a high BrdUTP pool is accessible to the replication apparatus. The specific sites of mutation further suggest that biased pools induced by BrdUrd in T4 bacteriophage include expansion of the BrdUTP pool and depletion of the dCTP pool. Lastly, we detected inhibition of CDP reduction by both BrdUTP and dTTP on T4 rNDP reductase. Comparing dNTP biosynthesizing enzyme activities in vitro and in vivo, Tomich et al (1974) found that the enzymes become active in T4 infected cells

considerably later than active enzymes can be detected in extracts of infected cells. They proposed that T4 DNA precursor biosynthesis enzymes become enzymatically active only after they are assembled into a dNTP-biosynthesizing multienzyme complex.

Based on our observations, we propose that the mechanism of mutagenesis stimulated by BrdUrd in T4 bacteriophage is as follows. After cells take up BrdUrd from the medium, thymidine kinase and other enzymes in the DNA biosynthesizing multienzyme complex phosphorylate BrdUrd to BrdUTP. The locally expanded BrdUTP pool, then, inhibits CDP reduction through feedback inhibition of T4 rNDP reductase, and this depletes the hm-dCTP pool around replication forks. Therefore, biased dNTP pool ratio induced by BrdUrd is BrdUTP to hm-dCTP, leading to predominant C-to-T transition.

How do dNTP-biosynthesizing enzymes other than rNDP reductase in a dNTP multienzyme complex participate in BrdUrd mutagenesis? Although the answer to this question is uncertain, one should consider that pyrimidine nucleotide pools are also controlled by other allosteric enzymes as well as rNDP reductase. The distinct allosterically regulated enzymes involved in BrdUrd mutagenesis in *E. coli* and T4 phage include thymidine kinase, deoxycytidylate deaminase and rNDP reductase (see Chapter I-3 and Table I-2). Although only rNDP reductase was replaced by host protein in our "metabolic engineering" experiment, such switching also altered protein-protein interactions, reflected in the dNTP synthetase complex formation (Moen et al 1988) and spontaneous mutation frequency (Table II-3). Therefore, understanding the effects of dNTP biosynthesizing multienzyme complex as well as individual proteins in this complex on overall dNTP pool regulations should be emphasized in future studies.

Acknowledgement

Financial support for this work came from NSF research grant no. DMB-8916366. We thank Dr. R. McParland for synthesis of the oligonucleotides and Mrs. L. Wheeler for capable technical assistance.

Table II-1. Bacterial and phage strains

Bacterial or phage strain		Genotype or phenotype
E. coli	B	Wild-type
	BB	Suppresses *rII* mutations
	K38(λ)	Restrictive host for *rII* mutants
	ED8689	sup^o, $hsdR^-$, $hsdM^+$ K12 strain
	ED8689/pBR322	ED8689 carrying pBR322 plasmid
	ED8689/pPS2	ED8689 carrying pBR322-derived plasmid containing the *nrd* A and *nrd* B genes of *E. coli*
T4	T4D	Wild-type
	nrd am A67	defective in the large subunit of rNDP reductase
	rII SN103	*rII* B^-, revertable to wild type by GC-to-AT transition
	rII UV215	*rII* A^-, revertable to wild type by AT-to-GC transition

Table II-2. Ribonucleotide reductase substitution and phage yields

	E. coli strain	
Phage Strain	ED8689/pBR322	ED8689/pPS2
T4D	1.00	1.02
T4 *am nrd* A67	0.18	0.81

Bacteria were grown in SM9 medium to a density of about 2 $X10^8$ cells/ml and infected by T4D or T4 *am nrd* A67 at a multiplicity of 6. Bacterial density was measured by spectrophotometer before infection, and phage yields were determined by plating on *E. coli B* cultures 90 min after infection at 37^0 C.

Table II-3. Effects of rNDP reductase on BrdUrd mutagenesis

		1	2	3	4
Phage *rII* genotype		UV215	UV215	SN103	SN103
Phage *nrd* A geneotype		+	*am* A67	+	*am* A67
Plasmid in host cells		pBR322	pPS2	pBR322	pPS2
rII reversion pathway		AT-to-GC	AT-to-GC	GC-to-AT	GC-to-AT
rNDP reductase		T4	Host	T4	Host
	[BrdUrd] (μM)		Revertants/10^8 phage		
Expt. 1	0	2.4	4.0	1.3	7.7
	100	1,740	10,000	15,100	2,130
Expt. 2	0	4.0	7.5	2.2	8.3
	100	3,960	21,700	31,500	4,850
			Relative revertant abundance		
Average	0	1.0	1.0	1.0	1.0
	100	858	2,693	12,970	430

Revertants/10^8 phage represents the relative titer of a phage lysate, plated on *Escherichia coli* strains K38(λ) and BB. All operations after addtion of BrdUrd were carried out in subdued light.

Table II-4. The partial DNA sequence of T4 *rII* SN103

CACAATCCGTCGTGTTTTGAAAAATGCTGAAGAAGCTAAACGCCCT
GTGTTAGGCAGCACAAAACTTTTTACGACTTCTTCGATTTGCGGGA

AAAGTTACTATTAGCGGTGATATTACAGTTA AAGTTAATAGCGAT
TTTCAATGATAATCGCCACTATAATGTCAAT TTCAATTATCGCTA

GCAGTTATTGCTCCAGTTGCTAAATCTGACATTATTTGGAATGCA
CGTCAATAACGAGGTCAACGATTTCGACTGA AATAAACCTTACGT

TCTAAAAAATTCATTCCAATTACTGTTGATGG CGTAACTTATA

AGATTTTTTAAGTAAGGTTAATGACAACTACC CGATTGAATAT

ACGCAACTCCTAATACTCA ---------- 3'
TGCGTTGAGGATTATGAGT---------- 5'

Only the DNA sequence from # 114 to # 310 from *rII* B translation start is shown. The shadowed GC base pair at #265 was identified as the mutation site for *rII* SN103 with an AT-to-GC transition from wild type. The underlining represents segments used in oligonucleotides used for template amplification and DNA sequencing.

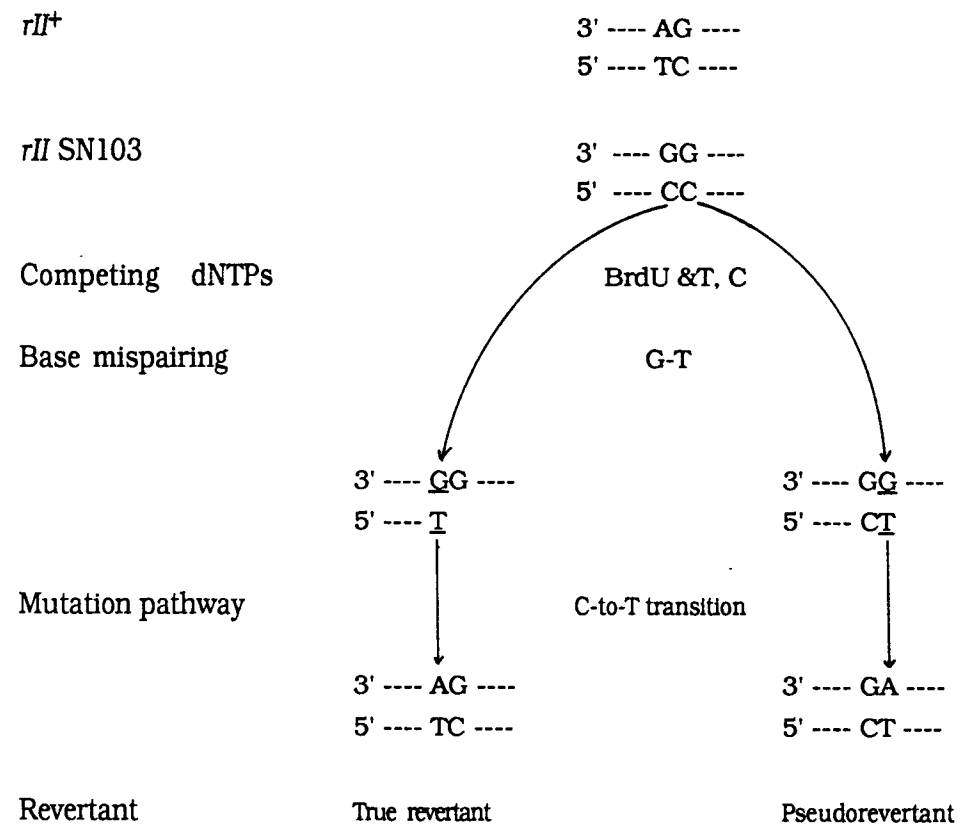
Table II-5. DNA sequence of mutations induced by BrdUrd

	DNA sequence[1]	Numbers observed	Amino Acid[2]	Mutation Pathway
Wild-type	ATT TCA ATT		Ser	
Mutant	ATT CCA ATT		Pro	
Revertants	ATT CTA ATT	4	Leu	GC-to-AT
	ATT TCA ATT	4	Ser	GC-to-AT
	ATT ACA ATT	1	Thr	GC-to-TA

[1] Part of the sense strand in *rII* B is shown from 5' to 3'. The mutated site in *rII* SN103 is a T-to-C transition at #265 from the translational start.

[2] Amino acid is that specified by the second of the three codons shown.

Table II-6. Putative mutation pathways stimulated by BrdUrd



The data are from Table II-5. Only GC-to-AT transitions were shown here, which represented 8 of 9 reversion mutations analyzed. Each mutable nucleotide and mismatch is underlined. The G-T mismatch underlined here includes both guanine-bromouracil and guanine-thymine mismatches.

Table II-7. Perturbations of dNTP pool ratios

	[BrdUrd] μM	[dATP+dTTP+BrdUTP] / [dGTP+dCTP+Hm-dCTP]	[purines] / [pyrimidines]
T4D/pBR322	0	1.15	0.69
	100	1.34	0.67
T4 *am nrd* A67/pPS2	0	0.72	0.96
	100	0.79	1.17

The original data for caculation of dNTP pool ratios are shown in Figure II-3.

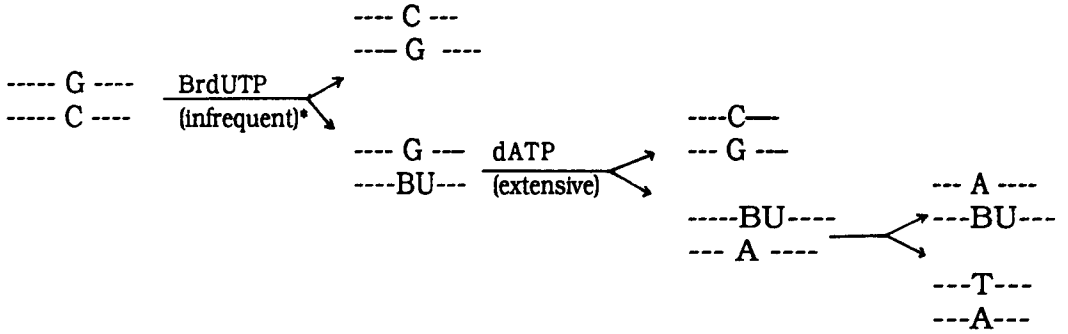
Figure II-1. Mutagenic pathways involving 5-bromodeoxyuridine 5'-triphosphate

A. Incorporation error occurs when BrdUrd triphosphate (BrdUTP) mispairs with a guanine residue in replicating DNA, which causes GC-to-AT transition. The crucial pool ratio for this pathway is [BrdUTP+dTTP]/dCTP. In T4-infected cells, dCTP pool is actual hm-dCTP pool (Mathews 1972).

B. Replication error occurs when a BrdUrd residue in replicating DNA mispaires with dGTP, which causes AT-to-GC transition. The crucial pool ratio for this pathway is that of dGTP pool to dATP pool.

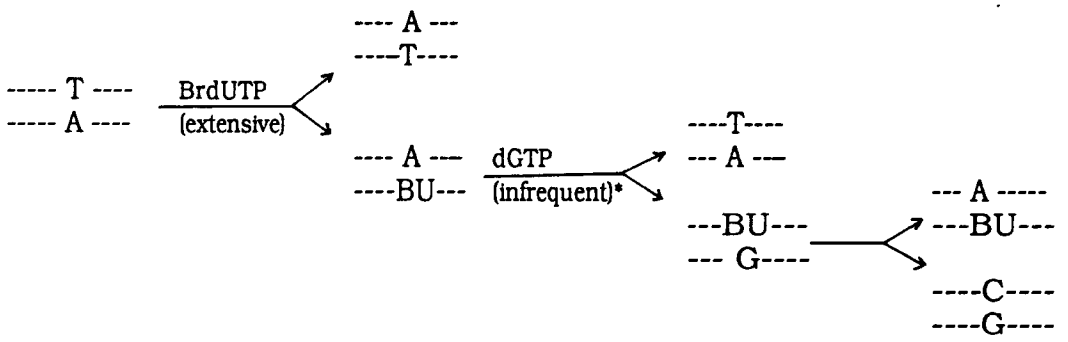
Figure II-1

A. Incorporation error



* BrdUTP and dTTP pool-, and dCTP pool-dependent step

B. Replication error



* dGTP pool- and dATP pool-dependent step

Figure II-2. DNA amplification and sequencing

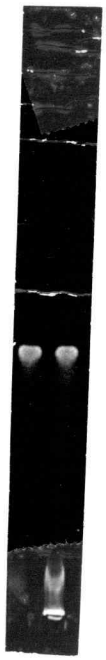
A. An agarose gel shown 200 bp PCR-amplified DNA segment at *rII* B locus.

B. DNA sequencing gel for PCR products. JJ2 primer was end-labelled with [γ-^{32}P]-ATP for sequencing. The arrows pointed out the mutation sites.

C. Two synthetic primers. JJ1 and JJ2 were designed based on the *rII* genetic map by Benzer et al (1961) and *rII* DNA sequence reported by Pribnow et al (1981). JJ1 and JJ2 are at oligonucleotide primers #114 to #133 of sense-strand and #291 to #310 of antisense strand from *rII* B translation start, respectively.

Figure II-2

A



B

C

JJ1 CAC AAT CCG TCG TGT TTT G

JJ2 TGA GTA TTA GGA GTT GCG T

Figure II-3. Effects of 5-bromodeoxyuridine upon dNTP pools

This enzymatic dNTP pool assay procedure cannot distinguish dTTP from BrdUTP and dCTP from hm-dCTP. However, early study in this laboratory showed that the dCTP pool is replaced completely by hm-dCTP within five minutes of T4D infection. Therefore, the detected dCTP pool in phage-infected cells here is actual hm-dCTP pool.

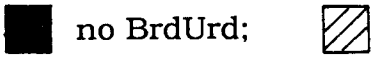


Figure II-3

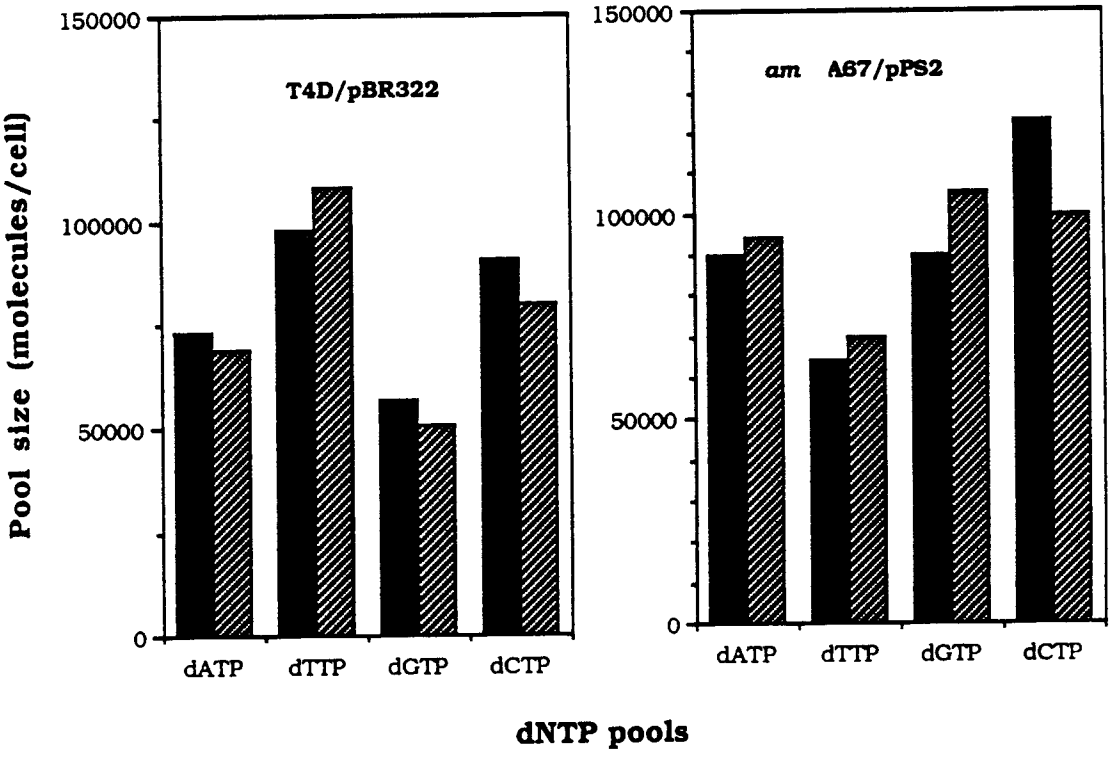

150000
100000
50000
0
T4D/pBR322
dATP
dTTP
dGTP
dCTP
150000
100000
50000
0
am A67/pPS2
dATP
dTTP
dGTP
dCTP
Pool size (molecules/cell)
dNTP pools

Figure II-4. Effect of dNTPs upon CDP reduction by T4 ribonucleotide reductase in crude extracts

The crude extract was incubated with reductase assay mixture at 37^{o} C for 10 minutes. The enzyme activity is expressed relative to the control treatment, which did not involve any extra dNTP added to the reaction mixture.

Figure II-4

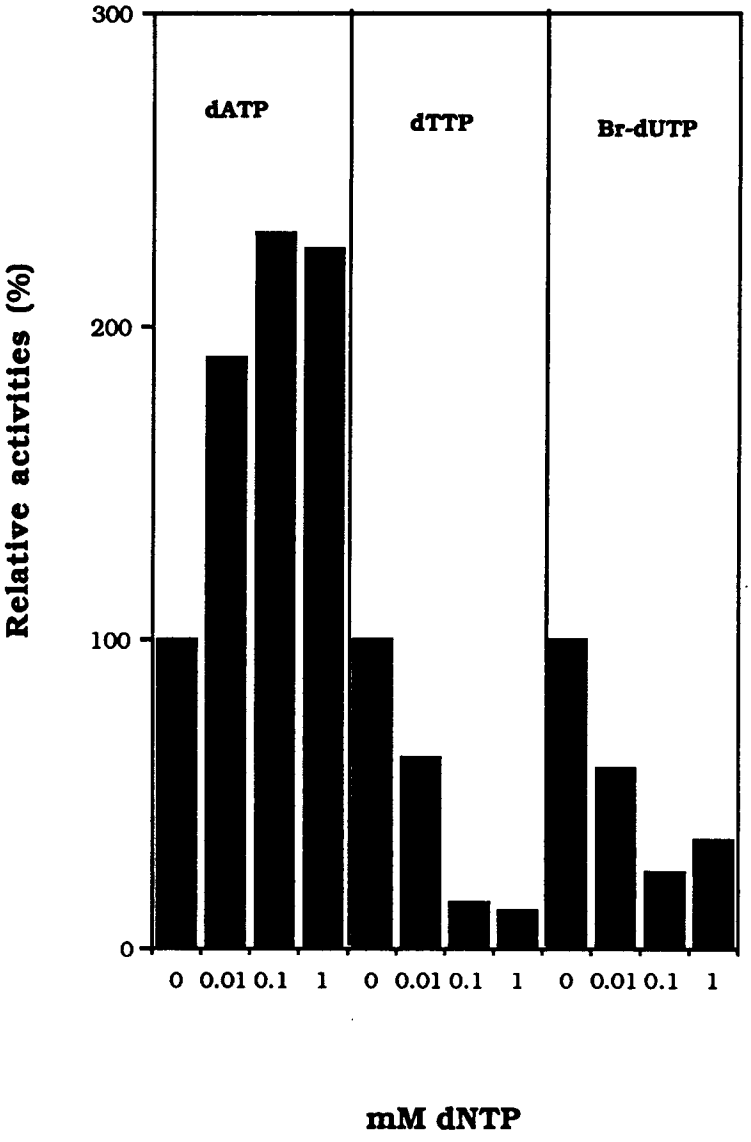

300
200
100
0
dATP
dTTP
Br-dUTP
Relative activities (%)
0 0.01 0.1 1 0 0.01 0.1 1 0 0.01 0.1 1
mM dNTP

Figure II-5. Control of T4 ribonucleotide reductase by dNTPs on CDP reduction

Two different enzyme preparations as indicated in the figure were used for reductase assay with conditions the same as those described for Figure II-4. The CDP reduction is inhibited by addition of 1 mM Br-dUTP or dTTP in crude extract, but not in partially purified enzyme preparation. The allosteric behavior of T4 CDP reductase in crude extract is opposite to that of earlier studies (Berglund 1972)

0, no dNTP added to the assay mixture;

B, 1 mM 5-Br-dUTP;

T, 1 mM dTTP;

A, 1 mM dATP.

Figure II-5

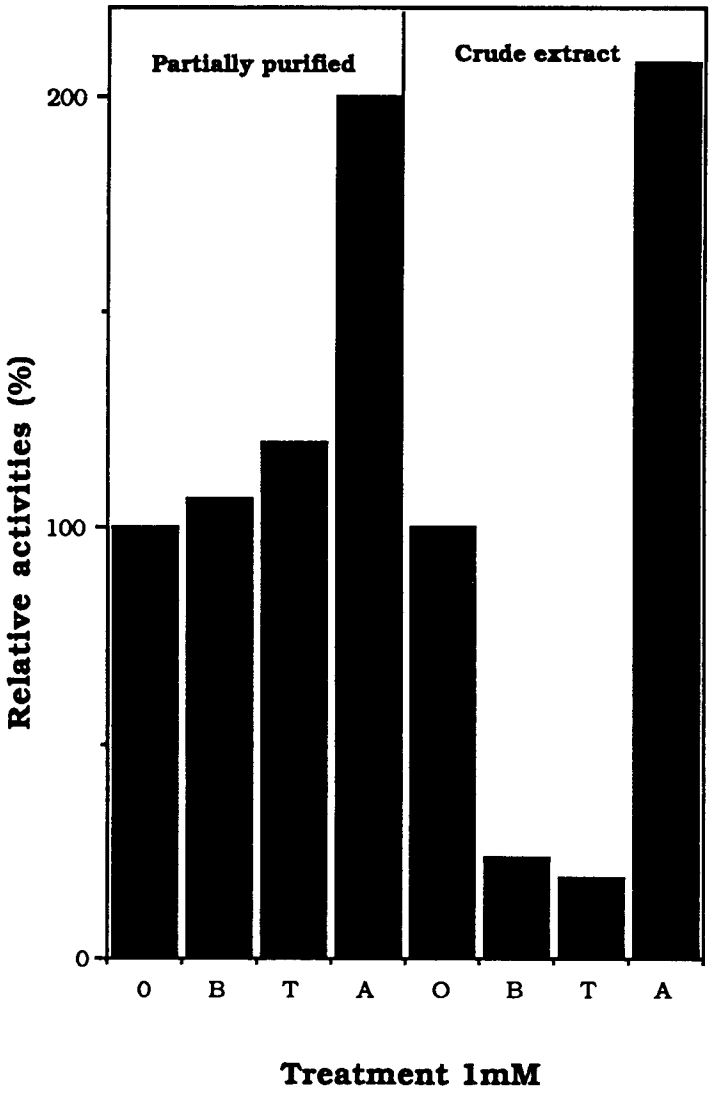
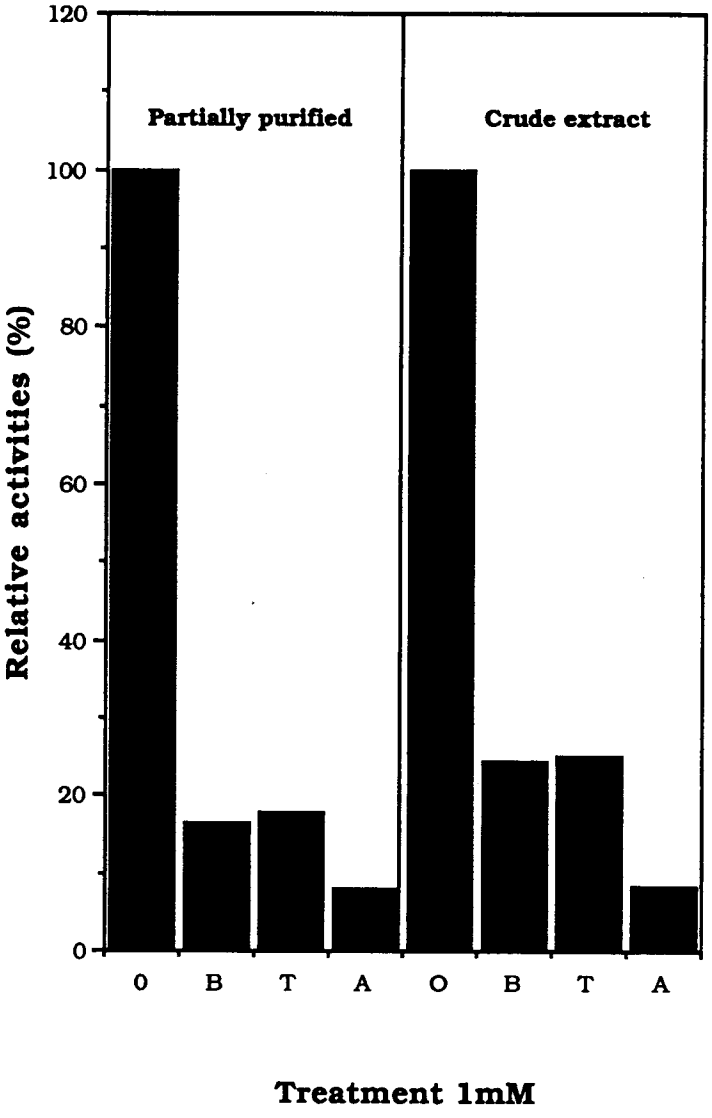

Partially purified
Crude extract
200
100
0
Relative activities (%)
0 B T A O B T A
Treatment 1mM

Figure II-6. Effect of dNTPs upon reduction of CDP by *E. coli* rNDP reductase

Enzymatic assay conditions were the same as those described for Figure II-5. Comparing this figure with Figure II-5, one can find that allosteric behavior of *E. coli* CDP reductase in both enzyme preparations is essentially the same as reported (Theland and Reichard 1979), supporting the notion that *E. coli* CDP reductase is a feed-back sensitive enzyme.

Figure II-6


120
100
80
60
40
20
0
Relative activities (%)
Partially purified
Crude extract
0
B
T
A
O
B
T
A
Treatment 1mM

Chapter III

Analysis of Mutagenesis Induced by a Thermolabile T4 Phage Deoxycytidylate Hydroxymethylase Suggests Localized Deoxyribonucleotide Pool Imbalance

Running title: Mutagenesis induced by *ts* dCMP hydroxymethylase

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1. Abstract

To understand the molecular basis of mutation stimulated by deoxyribonucleotide pool imbalance, we studied a temperature-sensitive T4 phage gene 42 mutant (*ts* LB3), which specifies a thermolabile deoxycytidylate hydroxymethylase. Analysis of *rII* mutations, revertible to wild type by either GC-to-AT or AT-to-GC transitions, showed 8- to 80-fold stimulations of GC-to-AT mutations at a semi-permissive temperature (34^{o} C). Eleven individual mutations induced by *ts* LB3 at the most highly revertible marker, *rII* SN103, were sequenced after amplification of the template by polymerase chain reaction. Four types of mutations were observed in toto, with both cytosines within one triplet (CCA) changing to either thymine or adenine. This is consistent with the hypothesis that hydroxymethyl-deoxycytidine triphosphate pools are depleted at replication sites. However, dNTP pool measurements in extracts of cultures at 34^{o} C showed no significant deviations from values obtained at 28^{o} C, suggesting that pool imbalances occur only locally, close to replication forks.

Our studies support the hypothesis that the mutator phenotype displayed by *ts* LB3 at a semi-permissive temperature is a consequence of perturbation of the flow of nucleotide precursors into the DNA replication machinery. Putative localized depletion of hm-dCTP increases effective dTTP/hm-dCTP and dATP/hm-dCTP pool ratios, resulting in the observed C-to-T transition and C-to-A transversion mutations.

Key Words: mutagenesis; replication fidelity; Deoxyribonucleotide pools; dCMP hydroxymethylase; T4 bacteriophage

2. Introduction

A crucial factor determining the accuracy of DNA replication is maintenance of a balanced supply of deoxyribonucleoside triphosphates (dNTPs) at replication sites. Perturbations of dNTP pools have deleterious genetic consequences, including increased mutagenesis, recombination, chromosomal abnormalities and cell death (de Serres, 1985, Kunz 1988, Meuth 1989). Although the precise mutagenic mechanisms are not yet well understood, the coordinated action of dNTP biosynthetic enzymes near replication sites may be involved in maintaining precursor balance.

In T4 bacteriophage, a system that has been extensively investigated, several DNA precursor biosynthesis enzymes interact to form a multienzyme complex (Chiu and Greenberg 1982, Mathews et al 1988, Moen et al 1988). While direct evidence for a linkage of this complex with replication sites has still not been obtained, there is reason to believe that this "dNTP synthetase complex" plays a role in delivering precursors to the replication apparatus. For example, studies of nucleotide incorporation in permeabilized cells support the existence of kinetically distinct pools of dNTPs at replication sites (Reddy and Mathews 1978). Moreover, disruption or disturbance of the complex with mutations affecting a dNTP biosynthetic enzyme often increases spontaneous mutation rates (Chiu and Greenberg 1973, Drake 1973, Sargent and Mathews 1987, Sargent et al 1989). These and other data (Mathews and Sinha 1982) suggest that dNTP concentrations around replication forks could be higher than at other sites in the cell.

Among T4 dNTP-synthesizing enzymes, deoxycytidylate hydroxymethylase has been shown to be a determinant of replication fidelity. dCMP hydroxymethylase, encoded by gene 42, converts dCMP

to 5-hydroxymethyldeoxycytidine monophosphate (hm-dCMP), which is subsequently converted to hm-dCTP for DNA replication. Early experiments showed that certain temperature-sensitive gene 42 mutations display a mutator phenotype at 34^{0} C, a semi-permissive temperature that only partially affects phage viability (Drake 1973, Chiu and Greenberg 1973). Williams and Drake (1977) further analyzed these mutations and found that GC-to-AT transition is the principal mutagenic pathway stimulated by *ts* LB3 at 34^{0} C (although T4 DNA contains hydroxymethylcytosine, or HMC, we use GC throughout this paper instead of G-HMC, for simplicity). These results were originally interpreted in terms of a direct involvement of dCMP hydroxymethylase in the replication process. Consistent with this interpretation is the fact that permeabilized *Escherichia coli* cells infected with gene 42 mutants cannot synthesize DNA *in vitro*, even when the metabolic block to DNA replication is bypassed by provision of hm-dCTP (Wovcha et al 1973, North et al 1976).

Once it was realized that dCMP hydroxymethylase functions as part of a multienzyme complex (Allen et al 1980, Chiu et al 1982, Moen et al 1988), the mutator phenotype of *ts* gene 42 mutations could be rationalized in terms of abnormal protein-protein interactions at restrictive or semi-permissive temperatures. In other words, the mutant protein might still be active in catalyzing the conversion of dCMP to hm-dCMP, but protein-protein interactions that control reaction fluxes in dNTP synthesis would be defective. In fact, we have obtained results of this type in vitro (Reddy and Mathews 1978, Thylén and Mathews 1989).

A more specific model would be localized deficiency of hm-dCTP at replication forks. If the multienzyme complex that synthesizes dNTPs is located near replication sites, and if its action is necessary to

replenish the pools of "replication-active" dNTPs, then studies of mutagenesis induced by a thermolabile dCMP hydroxymethylase could provide insight into the environment at replication sites with regard to dNTPs. Further, this model could explain why most mutations induced under these conditions are GC-to-AT transitions -- a pathway that is favored when hm-dCTP is deficient.

However, one must also consider a trivial explanation for the data, namely, that the thermolability of mutant hydroxymethylase is greater in vivo than in vitro. Under these conditions the total hm-dCTP pool would decrease, and GC-to-AT transitions should be favored regardless of the environment at replication sites. Since the earlier investigators did not carry out dNTP pool measurements, that was one goal of this study. Also, we wished to learn more about molecular events in mutagenesis by directly determining base sequences about mutant sites. We followed reversion at sites within *rII* locus, induced at semipermissive temperatures by T4 *ts* LB3, a *ts* gene 42 mutant. Our studies support the conclusion that the *ts* LB3 mutator phenotype results from localized deoxyribonucleotide pool imbalance around replication forks.

3. Materials and methods

Reagents

[5-^{3}H]-dCMP used for dCMP hydroxymethylase assay was purchased from Schwarz/Mann. [γ-^{32}P] rATP used for end-labelling of primer was obtained from New England Nuclear. [^{3}H]-labelled deoxyribonucleoside 5'-triphosphates used for dNTP pool assay, including [8-^{3}H]dATP, [methyl-^{3}H]dTTP, [5-^{3}H]dCTP and [8-^{3}H]dGTP, were purchased from ICN Pharmaceuticals. Ribonucleosides, deoxyribonucleosides, ribonucleotides and deoxyribonucleotides were purchased from Sigma. Other deoxyribonucleoside triphosphates, dideoxyribonucleoside triphosphates and copolymers poly (dA-dT) and poly (dI-dC) were purchased from Pharmacia. Tri-N-octylamine was from ICN Pharmaceuticals, and Freon-113 (1,1,2-trichlorotrifluoroethane) was from Aldrich Chemical Company.

Purified *E. coli* DNA polymerase I was purchased from Boehringer-Mannheim. *Taq* DNA polymerase was obtained from Promega. Polynucleotide kinase was from New England Biolabs. DNA sequenase (version 2.0) was from USB.

Primers were synthesized on a model 380B DNA synthesizer from Applied Biosystems Inc. (ABI) by Dr. R McParland of the Center for Gene Research and Biotechnology, Oregon State University.

Bacterial and phage strains

Strains used are listed in Table III-1. Double mutants of T4 *ts* LB3 X *rII* were prepared in this laboratory as follows (Hall et al 1967). Freshly prepared 1 ml of *E. coli* BB (2 X 10^{8} phage/ml)

was co-infected with *rII* mutant and *ts* LB3 mutant at a multiplicity of 4 each. *rII* genotype was identified based on different phenotype when plated on *E. coli* BB and *E. coli* K38(λ) (Benzer 1961). *ts* gene 42 phenotype was identified on sensitivity to temperatures (Drake 1973).

rII reversion assay

rII reversion frequency of each double mutant of *rII* with *ts* gene42 mutation at different temperatures was measured essentially as described by Drake (1973). Fresh *E. coli* BB cells were grown to a density of 2.0 X 10^8 cells per ml at the desired temperatures and infected with double mutants. Phage-infected cells were incubated at the pre-infection growth temperatures as indicated. The infections were stopped by addition of a few drops of chloroform, and phages in each lysate were plated on nutrient broth plates containing either *E. coli* BB or *E. coli* K38(λ) at room temperature for determining the revertant fraction.

Deoxycytidylate hydroxymethylase assays

E. coli B cultures were grown at ambient temperature in SM9 medium, to a density of about 3 X 10^8 cells/ml. After addition of L-tryptophan to 20 μg/ml, the cells were infected with *ts* LB3 at about six phages per bacterium. At twenty minutes after infection at room temperature, the phage-infected cells were harvested by rapidly chilling the cells on ice and centrifugation. The cell pellets were resuspended in cool 0.5 M Tris-HCl buffer, pH 7.8. and treated sonically with a sonicator 3 times for 10 seconds in the ice bath. The supernatant of the crude extract was used for enzyme assays after centrifugation at 12,000

X g for 10 minutes as described by Wang (1989). 100 µl reaction buffer containing 60 mM KH_2PO4, pH 7.4, 29.4 mM 2-mercaptoethanol, 2.5 mM *R, S* tetrahydrofolate, 2 mM Na_3EDTA, and 0.2 µM 5-fluorodeoxyuridylate, was mixed with 50 µl enzyme extract and 30 µl H_2O. The reaction was initiated by adding 20 µl of 30 mM [5-^{3}H]dCMP, and incubation continued at the indicated temperatures for 15 minutes. Each reaction was terminated by adding with vigorous mixing an equal volume of stop mixture containing 15% activated charcoal in 4% trichloroacetic acid. After removal of the charcoal-adsorbed nucleotides by centrifugation, the radioactivity of the [^{3}H]H_2O generated in the reaction was measured by counting an aliquot of supernatant in a scintillation counter.

dNTP pool determinations

25-ml *E. coli* B cultures were grown at different temperatures as indicated to a density of 3 X 10^8 cells/ml in SM9 medium. After addition of L-tryptophan to 20 µg/ml, the cells were infected with *ts* LB3 at about six phages per bacterium. Uninfected cultures were treated identically except that no phage were added. The cultures were harvested by rapid filtration at the indicated times after infection. dNTP pools were extracted with trichloroacetic acid and neutralized with 0.5 M tri-N-octylamine/Freon as described by Sargent and Mathews (1987). Copolymer poly (dA-dT) or poly (dI-dC) templates and purified DNA polymerase I were used for measuring dNTP pools in cell extracts as reported by Sargent and Mathews (1987) with minor modifications. The 100-µl reaction mixture contained 45 mM Tris, pH 8.3, 4.5 mM $MgCl_2$, 1 mM mercaptoethanol, 1 µM [^{3}H]dNTP (10 mCi/µmole)

complementary to the dNTP being assayed, 1 unit/ml *E. coli* DNA polymerase I, 20 μM template, 0.2 mg/ml bovine serum albumin, 100 mM dAMP and 10 μl of dNTP standard or cell extract. The dNTP pools are expressed as number of dNTP molecules per cell.

Template amplification and DNA sequencing

Two primers for template amplification and DNA sequencing, JJ1 and JJ2, were designed based on the *rII* genetic map by Benzer et al (1961) and *rII* DNA sequence data reported by Pribnow et al (1981). JJ1 (CAC AAT CCG TCG TGT TTT G) and JJ2 (TGA GTA TTA GGA GTT GCG T) are at oligonucleotide primers #114 to #133 of sense-strand and #291 to #310 of antisense strand from *rII* B translation start, respectively. The primers were synthesized on an ABI 380B DNA synthesizer, and purified by 20% polyacrylamide gel electrophoresis and desalted with C_{18} Sep-Pak cartridges (Maniatis et al 1982). Phage particles were concentrated with ultracentrifugation or polyethylene glycol precipitation. Phage DNA was extracted with phenol/chloroform procedures (Maniatis et al 1982). DNA derived from 10^8 phage particles was subjected to amplification with 2 units of *Taq* DNA polymerase, as described by Saiki et al (1988). The reaction mixture, including 50 pmoles of each primer, was subjected to 40 cycles each with 1 minute at 93^{o} C, 2 minutes at 40^{o} C, and 2 minutes at 70^{o} C, by using a TwinBlock Thermocycler from Ericcomp Inc. PCR products were visualized by electrophoresis through 1.5% regular agarose or 1% regular agarose with 3% NeuSieve agarose gel. Unincorporated nucleotides and primers were removed with a Centricon-30 microconcentrator (Amicon). Sanger's DNA sequencing method was used for direct sequencing PCR products as described by Higuchi et al

(1988) with minor modification. Oligonucleotide primers were 5' end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase as described by Maniatis et al (1982). The end-label reaction mix contained 10 units of polynucleotide kinase, 10 pmoles of primer, 10 pmoles of [γ-^{32}P]ATP, 0.01 M $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, 0.01 mM EDTA, and 0.05 M Tris-HCl, pH 7.6. The reaction mixture was incubated at 37^{o} C for 45 minutes, then at 90^{o} C for 2 minutes. This end-labeled primer was heated to 90^{o} C for 5 minutes with PCR-amplified DNA, then immediately stored on ice. Each sequencing reaction was initiated by adding 2.8 µl of this mixture to 3.25 µl of dideoxy A, T, G, and C reaction mixtures composed of reagents provided in the "sequence kit" as follows: 2.5 µl of the A, T, G and C "termination mixes", 0.15 µl of sequenase (1 unit), 0.38 µl of "5X buffer", and 0.22 µl of 0.1 M DTT. After a six-minute incubation at 37^{o} C, each reaction was stopped by adding 4 µl of 95% (v/v) formamide/20 mM EDTA, then, each reaction mixture was heated to 85^{o} C for 2 minutes, and loaded into a 7% polyacrylamide gel. The sequencing gel was run at approximately 40 watts for 2 hours, dried and radioautographed with Kodak X-omat film at -70^{o} C.

4. Results

Temperature dependence of biological parameters

In order to establish an appropriate semi-permissive temperature for studying the thermolabile dCMP hydroxymethylase mutant, we measured dCMP hydroxymethylase activity and phage plating efficiency at different temperatures from 28° C to 43° C, as shown in Figures III-1 and III-2. Phage viability was more severely affected by temperature than was enzyme activity in extracts; we saw 42% loss of enzyme activity at 34° C and 70% loss of plaque-forming titer, while at 37° C the corresponding figures were 65% and 97%, respectively. Survival of *rII-ts* LB3 double mutants showed the same temperature dependence as seen with the *ts* LB3 single mutant.

Reversion of rII mutations at 34° C

The above data, plus previous studies (Chiu and Greenberg 1973, Williams and Drake 1977) indicated that 34° C was a suitable semipermissive temperature for our studies of mutagenesis stimulated by the thermolabile *ts* LB3 dCMP hydroxymethylase. Therefore, for several *rII-ts* LB3 double mutants we compared reversion rates at *rII* loci at 28° C and 34° C, as shown in Table III-2. One mutant, *rII* UV215, which should revert to wild type by AT-to-GC transition, showed no stimulation of mutation at 34° C. However, all other markers tested, all of which should revert to wild type by GC-to-AT transitions, were stimulated 8- to 80-fold at 34° C. The most sensitive marker tested, *rII* SN103, showed 8-fold stimulation of reversion at 32° C

(data not shown). These results are consistent with the conclusions of Williams and Drake (1977), namely, that the *ts* LB3 mutator specifically induces GC-to-AT transitions. The different markers tested show a tenfold range of reversion frequencies, suggesting a sequence context factor for LB3 mutagenesis.

dNTP pools

Since dCMP hydroxymethylase plays an essential role in synthesizing hm-dCTP, and since mutagenesis by a faulty dCMP hydroxymethylase causes GC-to-AT transitions, it seems likely that mutagenesis results from depleted pools of hm-dCTP. Are the putative depletions localized at replication sites, or do they extend throughout the cell? To approach this we carried out dNTP pool measurements, using two different protocols. First, as shown in Figure III-3, we initiated infection under normal conditions (28^{o} C), and at twenty minutes after infection raised temperatures to the indicated values, harvesting cells for pool determinations at the indicated times, and at forty minutes after infection returned cultures to the original 28^{o} C. Under these conditions no significant dNTP pool abnormalities were observed after shiftup either to 30^{o} C or 34^{o} C. At 37^{o} C slight dATP and dTTP pool expansions were seen, and at 43^{o} C, where viral DNA replication is effectively abolished (Mathews, 1972), all dNTP pools except hm-dCTP expanded, up to fifteen-fold. All of these changes were rapidly reversible upon return of the cultures to 28^{o} C.

As noted above, the shiftup protocol showed no dNTP pool imbalances at 34^{o} C where the mutant dCMP hydroxymethylase is known to exert a mutator effect. However, we must consider the possibility that pre-existing pools might obscure small changes in pool

size occurring after temperature shiftup. Therefore, we infected cells at a range of temperatures, maintaining constant temperature in each culture until cells were harvested, either at ten or twenty minutes after infection (Figure III-4). Again, while dATP and dTTP pools expanded at the higher temperature, no significant changes in hm-dCTP pools were noted over the range from 30^{o} C to 43^{o} C. As seen in our previous study (Mathews 1972) dGTP pools did not expand substantially under this condition, even when DNA replication was totally blocked, at 43^{o} C.

Nucleotide sequence of mutations

The classification of *rII* markers studied, in terms of reversion pathways, originally involved indirect methods, namely, the responses of mutants to chemical mutagens (Drake 1963). Now that mutant sites can be readily sequenced following PCR amplification, it is both possible and essential to gather direct information about mutagenic pathways, by sequence determination before and after mutagenesis. Our goal was to identify the sequence alteration responsible for the *rII* SN103 mutation and to determine the nucleotide sequences associated with about a dozen reversion and/or pseudoreversion events. Revertants were isolated from lysates of *E. coli* BB cultures infected either at 28^{o} C for twenty minutes followed by a shift to 34^{o} C, or at 34^{o} C throughout the infective cycle; recall that neither infection condition led to significant dNTP pool imbalances. The *rII* SN103 mutation was selected because it was the most sensitive of the various *rII* alleles tested to the effects of the mutator hydroxymethylase. This increases the likelihood that revertants and pseudorevertants will have arisen specifically under the influence of the mutator enzyme.

Figure III-5 shows details of the PCR amplification employed, including design of the primers and a typical sequence determination. The sequence data obtained are summarized in Table III-3. The *rII* SN103 mutant site is at nucleotide #265 from the *rII* B translation start and involves an AT-to-GC transition. When we sequenced 11 revertants stimulated by *ts* LB3 at 34^{0} C, we found that hydroxymethylcytosine either at the mutant site or its immediate 3' neighbor could undergo change either to thymine or adenine. Four types of revertants and pseudorevertants were observed in toto. Only one of the eleven sequenced was a true reversion, a GC-to-AT transition at the mutant site. The others were pseudorevertants, GC-to-AT transitions and GC-to-TA transversions with equal frequency.

5. Discussion

Are there localized dNTP pools at replication sites?

Several lines of evidence support a model of prokaryotic DNA replication in which dNTPs are functionally compartmentalized (Reddy and Mathews 1978, Manwaring and Fuchs 1979, Mathews and Sinha 1982). According to this model, DNA replication is supplied primarily by small, rapidly replenished pools that are located close to replication sites, while the much larger, more highly dispersed pools are more remote from replication sites, and hence, turn over more slowly. Because the pools are not physically separated, their existence and behavior must be analyzed by indirect methods. The point of this study was to ask whether the partial impairment of dCMP hydroxymethylase would exert its mutagenic effect by selectively depleting one replication-active pool, that of hm-dCTP. Our data are consistent with that interpretation. The total hm-dCTP pools measured in *ts* LB3 mutant infections were insensitive to temperature upshifts into the range where mutagenesis was greatly enhanced (Figure III-6). However, as discussed below, all of the mutational events observed can be explained as consequences of selective hm-dCTP pool depletion. We suggest that local concentrations of hm-dCTP were diminished in a 34^{o} C infection, but that these depletions cannot be seen, because of the backdrop of the much larger pools of hm-dCTP that are not localized at replication sites and that turn over slowly.

While this question cannot be answered with certainty, one can semiquantitatively describe dNTP pool turnover at T4 replication sites. The average volume of an *E. coli* cell is about 10^{-15} liters (Neidhardt 1987), and a T4-infected cell has 60 replication forks (Werner 1968).

The effective concentration of each dNTP is at least 200 μM at replication sites (Mathews and Sinha 1982). If we assume that the "immediate vicinity" of the 60 replication forks constitutes one per cent of the volume of the cell, this means that the replication-active hm-dCTP pool at any instant constitutes about 1200 molecules. At the same time, the total incorporation of hm-dCTP into DNA can be calculated at about 14,000 molecules per second per cell, if each of the 60 forks contains two chains, each growing at 700 nucleotides per second (McCarthy et al 1976), of which 16.5% are HMC nucleotides. Thus, the "replication-active" pool is seen to turn over about ten times per second. Even if some of the assumed numbers are incorrect by as much as an order of magnitude, it is evident that a partial impairment of hm-dCTP production could significantly diminish hm-dCTP concentrations at replication sites, without significantly affecting the overall hm-dCTP pool size.

Mutagenic pathways stimulated by mutator dCMP hydroxymethylase

As noted in Table III-3, the eleven revertants and pseudorevertants that we sequenced involved GC-to-AT transitions and GC-to-TA transversions. Both of these events could occur as the result of hm-dCTP pool depletion, as schematized in Table III-4. We postulate that the GC-to-AT transition occurs via formation of a G-T mismatch, where T and C compete for incorporation opposite template G. Similarly, we see the transversion as involving competition between C and A at a template G, with formation of a G-A mismatch.

Apparently, there is a base sequence context to mutagenesis,

because of the wide range of responses to the *ts*LB3 mutator that we saw among the four revertible *rII* mutations that we tested. While the total number of mutations that we sequenced is not sufficiently large for definitive conclusions about the sequence context, we note that the following observation could be significant for understanding the nature of the *ts* LB3 mutator phenotype. First, five of the six GC-to-AT transition mutations that we sequenced changed a G that was immediately 3' to a G. This suggests that proofreading, or a next-nucleotide effect (Fersht 1979), might contribute toward the sequence context. A purine-pyrimidine mismatch at the first G might be more efficiently proofread than that at the second, because the hm-dCTP pool depletion would slow down the incorporation of the next nucleotide and increase the mean residence time of a nucleotide misincorporated opposite the first G, as compared with the second G. Secondly, although a purine-purine mismatch is much more unstable than a purine-pyrimidine mismatch from the thermodynamic point of view (Perrino and Loeb 1989), half of the mutations (five of eleven) we sequenced are GC-to-TA transversions. High rates of transversion mutations in vivo through purine-purine mismatch have also been observed in analysis of mutations induced by a mammalian thy$^-$ mutation, a mutator condition that results from a different type of dNTP imbalance (Meuth 1989), suggesting that an A-A mismatch could be inefficiently corrected by proofreading or repair systems. Thirdly, as a consequence of depletion of hm-dCTP pool around replication sites, enhanced dNTP pool ratios should include that of dGTP to hm-dCTP, which will stimulate C-to-G transversion mutation through G-G mismatch. However, we did not observe any C-to-G transversion among eleven mutants. It is unclear whether such guanine-guanine mismatches are proofread or repaired more efficiently, or whether a C-to-G transversion in this codon is

unacceptable missense for the *rII* gene.

However, to make any clear statements about the sequence context for mutagenesis induced by dNTP perturbations will require the use of a forward mutation assay, where we can investigate a whole mutation spectrum and a large number of sequence contexts. Such an assay system is described in the next chapter.

Acknowledgement

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Table III-1. Bacterial and phage strains

Bacterial or phage strain		Genotype or phenotype	Source
E. coli	B	Wild-type	Our collection
	BB	Suppresses *rII* mutations	Our collection
	K38(λ)	Restrictive host for *rII* mutant	D. Pribnow
T4	T4D	Wild-type	Our collection
	ts LB3	*ts* gene 42 mutant, specifies a thermolabile dCMP hydroxy-methylase	W. B. Wood
	rII SN103	*rII* B^-	I. Tessman
	rII UV215	*rII* A^-	J. Drake
	rII UV363	*rII* B^-	J. Drake
	rII UV7	*rII* A^-	J. Drake
	rII SM94	*rII* B^-	J. Drake

Table III-2. Effect of ts^{42} mutation on reversion of *rII* mutations

rII mutation	likely reversion pathway	revertant fraction x 10^8					
		wild-type background			ts^{42} background		
		34° C	28° C	ratio	34° C	28° C	ratio
UV215	AT-to-GC	3	3	1.0	4	5	0.8
UV7	GC-to-AT	21	12	1.8	140	13	10.8
UV363	GC-to-AT	2	3	0.7	26	3	8.7
SM94	GC-to-AT	12	13	0.9	206	16	12.9
SN103	GC-to-AT	3	3	1.0	320	4	80

Revertant fraction is the relative plating efficiency at 30° C on *E. coli* K38(λ) as compared to *E. coli* BB. Each reversion assay involved counting at least triplicate plates.

Table III-3. DNA sequence of *rII* SN103 revertants induced in ts^{42} background

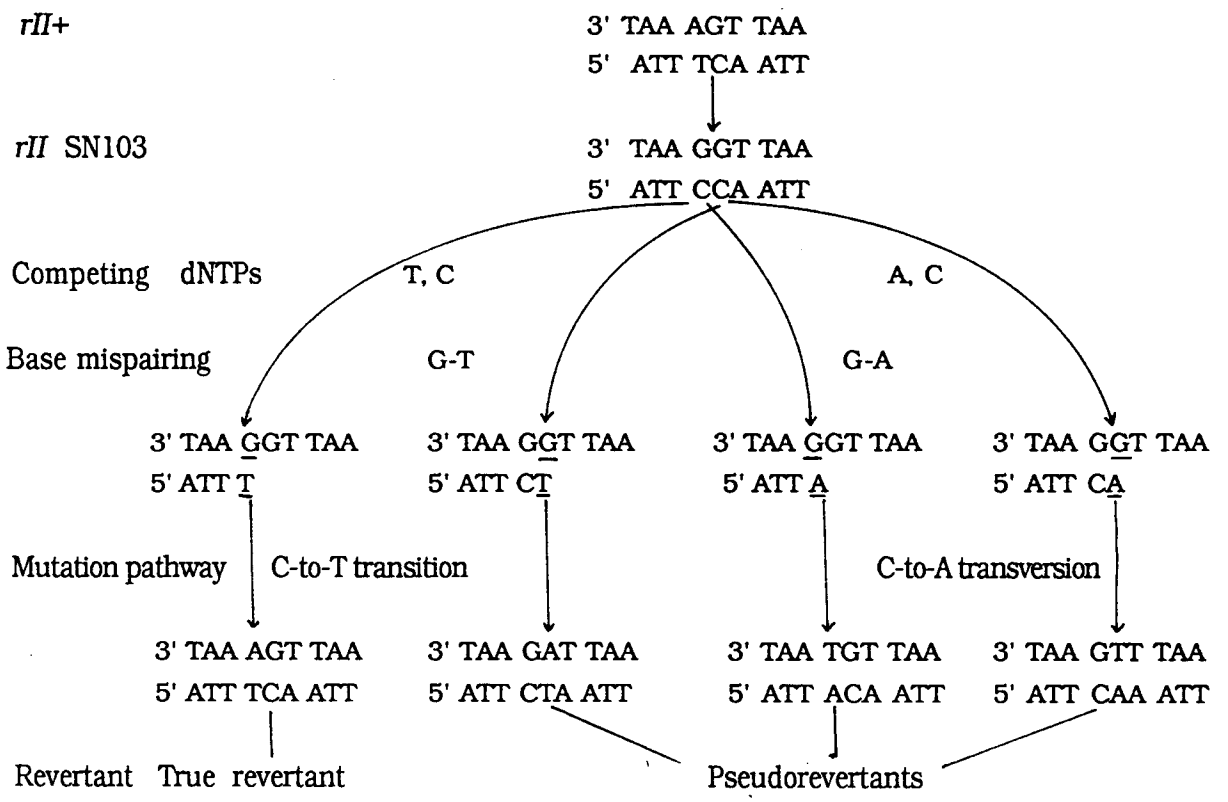
	DNA sequence[1]	Numbers observed			Amino Acid	Mutation Pathway
		A[2]	B[3]	Σ		
Wild-type	ATT TCA ATT				Ser	
Mutant	ATT CCA ATT				Pro	
Revertants	ATT CTA ATT	3	2	5	Leu	GC-to-AT
	ATT CAA ATT	1	1	2	Gln	GC-to-TA
	ATT TCA ATT	0	1	1	Ser	GC-to-AT
	ATT ACA ATT	1	2	3	Thr	GC-to-TA

1 Part of the sense strand in *rII* B is shown from 5' to 3'. The mutated site in *rII* SN103 is a T-to-C transition at #265 from the translational start.

2 Revertants from condition in which temperature was shifted to 34° C 20 minutes after infection with *ts* LB3 at 28° C.

3 Revertants formed when the temperature was held constant at 34° C. Amino acid is that specified by the second of the three codons shown.

Table III-4. Proposed mutation pathways stimulated by *ts* LB3[1,2]



[1] The data are from Table III-3.

[2] Each mutable nucleotide and mismatch is underlined.

Figure III-1. dCMP hydroxymethylase activity in extracts of *E. coli* B as a function of temperature of infection

Figure III-1

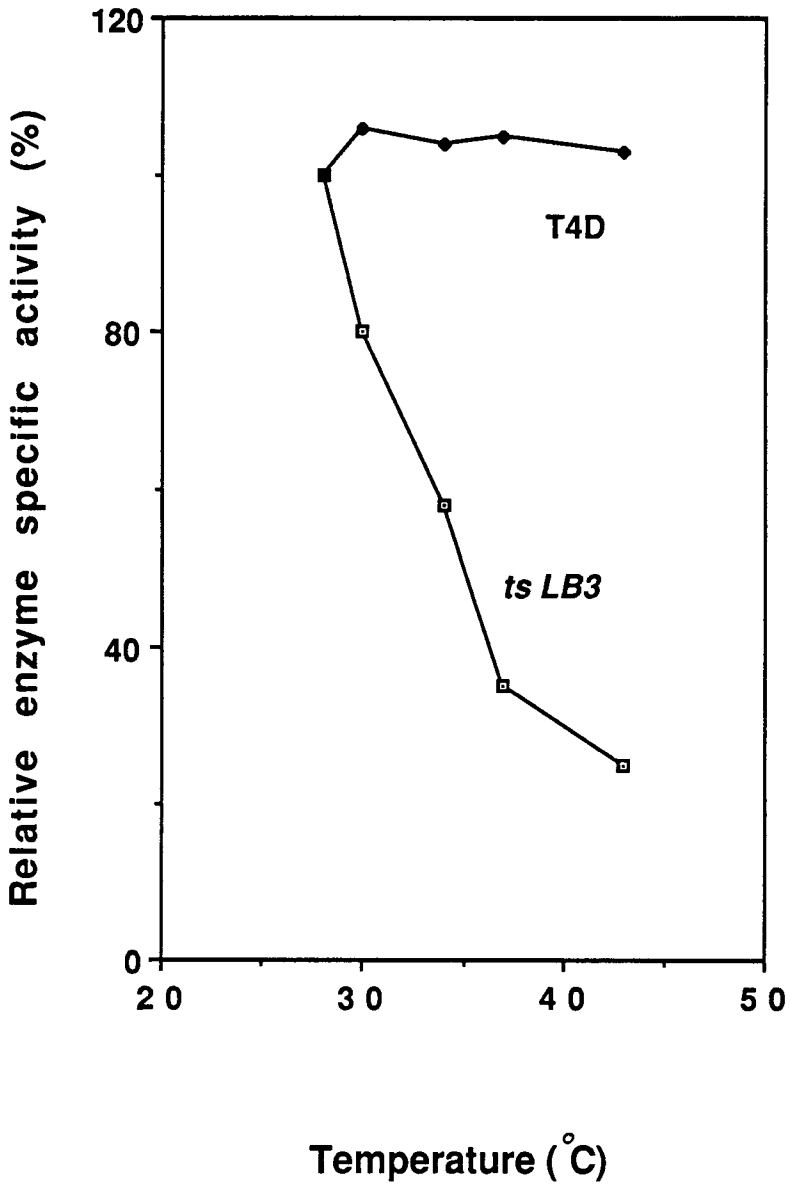

120
80
40
0
20
30
40
50
T4D
ts LB3
Relative enzyme specific activity (%)
Temperature (°C)

Figure III-2. Phage plating efficiency as a function of temperature

E. coli BB was used as plating host. Plaques were counted and recorded as percentage of the titer at 28^{o} C after incubation overnight at indicated temperatures.

Figure III-2

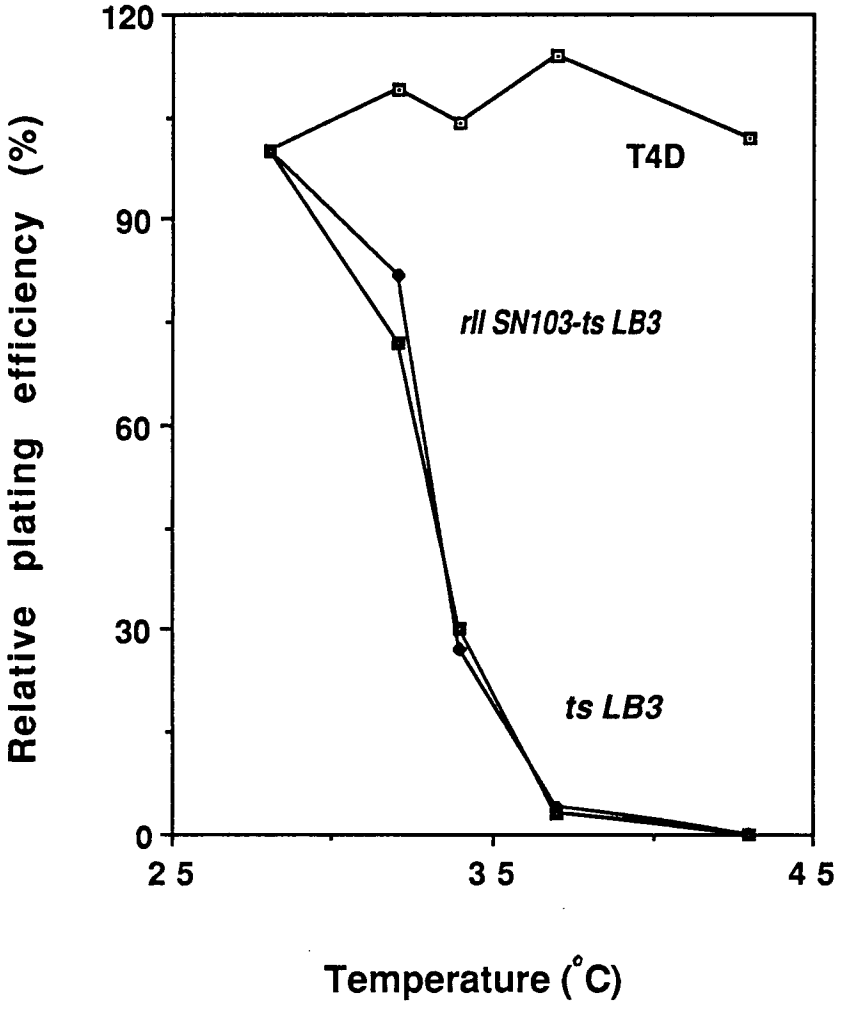

120
90
60
30
0
Relative plating efficiency (%)
T4D
rII SN103-ts LB3
ts LB3
25
35
45
Temperature (°C)

Figure III-3. Effect of temperature shift on dNTP pools

E. coli B was infected at 28^{0} C by *ts* LB3 at a multiplicity of 6 for 20 minutes, then shifted to the indicated temperatures: ◆ 30^{0} C, ▫ 34^{0} C, ♦ 37^{0} C, or ■ 43^{0} C for another 20 minutes, Finally, the temperature was shifted back to 28^{0} C. The times of temperature shift are shown in the figure with the arrows. In uninfected cells, there is no hm-dCTP, and the data points here reflect the dCTP content.

Figure III-3

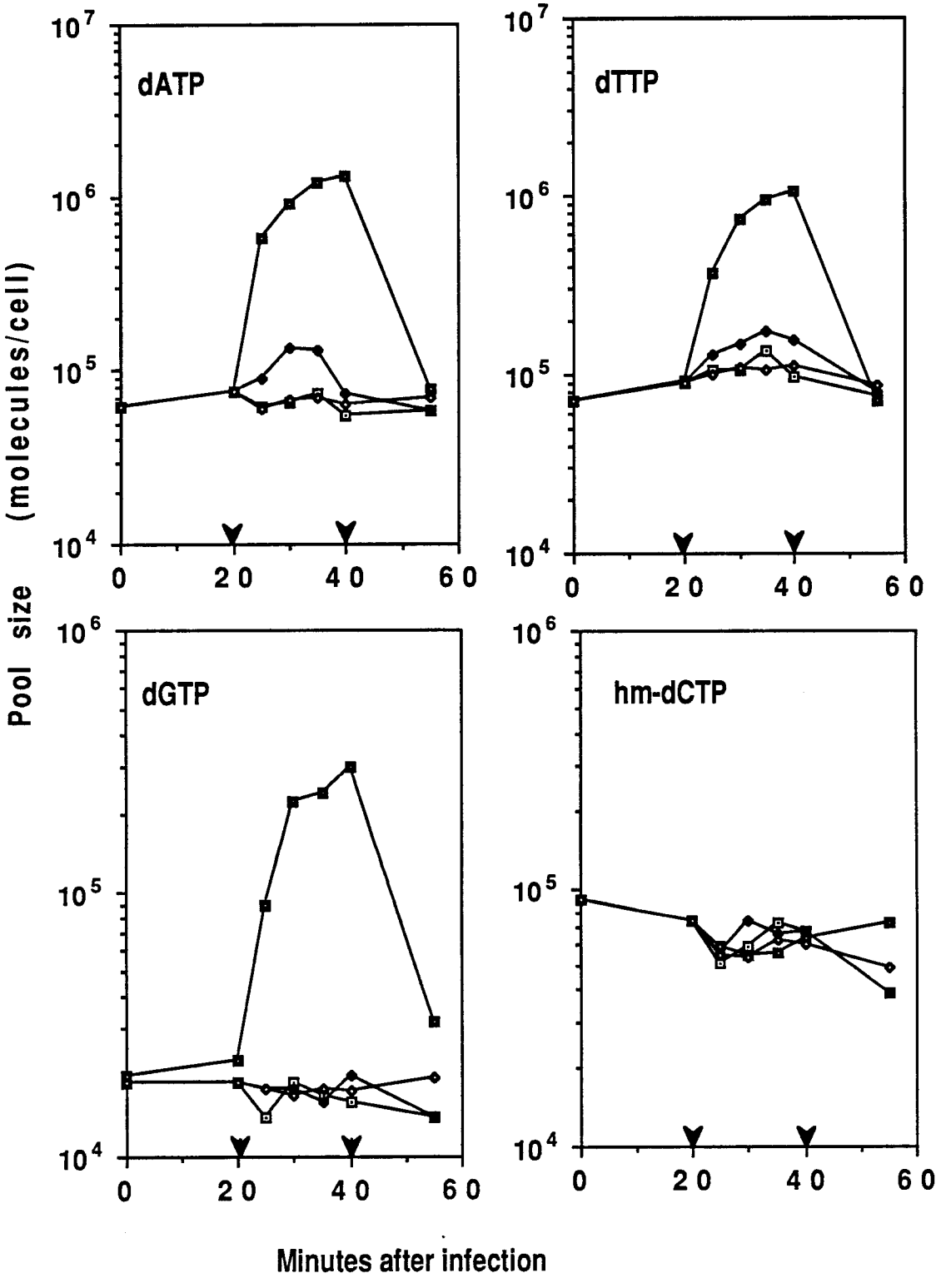

dATP
dTTP
dGTP
hm-dCTP
Pool size (molecules/cell)
Minutes after infection
10^7
10^6
10^5
10^4
0
20
40
60

Figure III-4. dNTP pools in *ts* LB3-infected *E. coli* B maintained at 34° C

A. Cells harvested after 10 minutes of infection

B. 20 minutes of infection

Although our dNTP assay procedure does not distinguish dCTP from hm-dCTP, we have shown (Mathews 1972) that the dCTP pool of *E. coli* is replaced completely by hm-dCTP within five minutes of T4D infection. Therefore, in Figures III-3, -4 and -6 we identify dCTP as the only cytosine dNTP in uninfected bacterial and hm-dCTP as the only one in infected cells.

Figure III-4

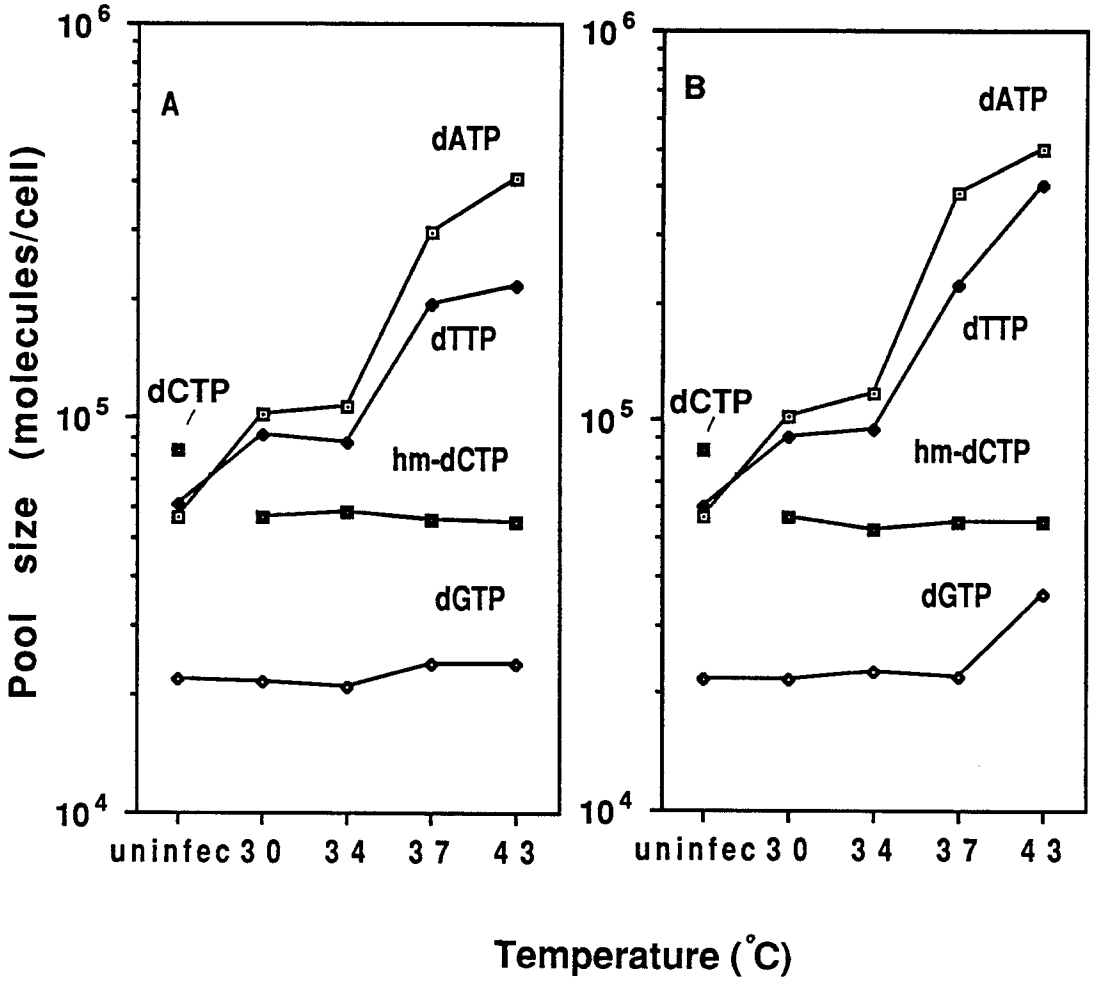

Pool size (molecules/cell)
10^6
10^5
10^4
A
dATP
dTTP
dCTP
hm-dCTP
dGTP
B
uninfec 30 34 37 43
Temperature (°C)

Figure III-5. DNA amplification and sequencing

A. Amplification of genomic DNA at *rII* B locus by PCR.

a, T4D;

b, *rII* SN103;

c, *rII* SN103-*ts*LB3;

d, revertant of *rII* SN103-*ts* LB3.

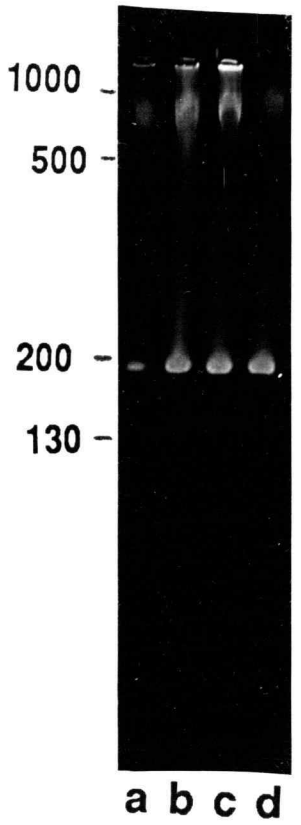
B. DNA sequencing gel for PCR products. JJ2 primer was end-labeled with [γ-^{32}P]-ATP for sequencing. The arrows indicated the mutated sites.

Lane 1, *rII* SN103;

Lanes 2-5, revertants of *rII* SN103-*ts* LB3.

Figure III-5

A



B

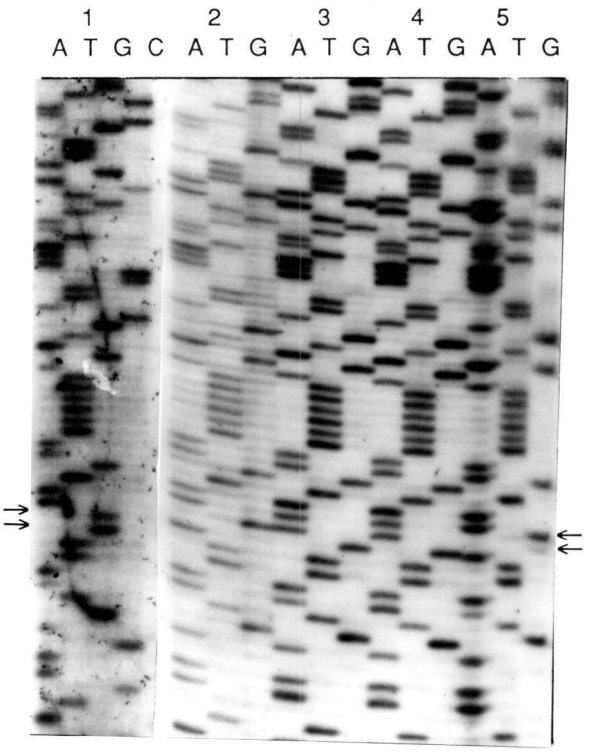
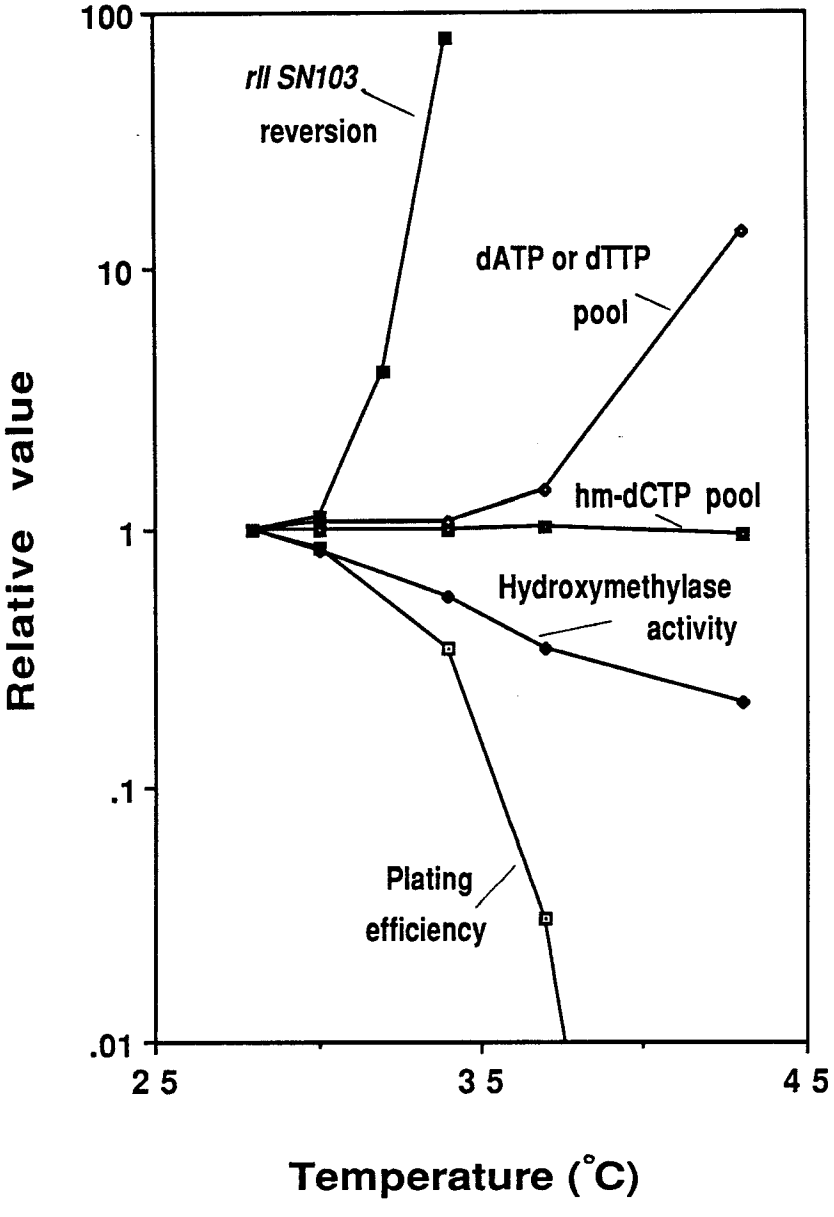


Figure III- 6. Temperature sensitivity of biological parameters in the *ts*^{42} mutator background

Data for this figure are from those of Figure III-1, -2, and -3, and Table III-2.

Figure III-6


100
10
1
.1
.01
Relative value
rII SN103
reversion
dATP or dTTP
pool
hm-dCTP pool
Hydroxymethylase
activity
Plating
efficiency
25
35
45
Temperature (°C)

Chapter IV

A Forward Mutation Assay System in Phage T4: Application to Gene 42 Mutator Mutations

Running title: Mutagenesis induced by *ts* gene 42

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1. Abstract

A forward mutation approach was adopted to study mutagenic specificity induced by bacteriophage T4 *ts* LB3, a mutant which encodes a thermolabile deoxycytidylate hydroxymethylase. The approach involves analyzing mutations induced by *ts* LB3 at a semi-permissive temperature (34^{o} C) in *tk*, the thymidine kinase gene. These were selected under near-ultraviolet light on synthetic agar plates containing bromodeoxyuridine. The thymidine kinase-negative mutant phenotype was confirmed by enzyme activity assays of phage-infected cell extracts. The thymidine kinase gene in the selected *tk*$^{-}$ mutants was amplified by polymerase-chain-reaction with two primers, located upstream and downstream of the translation start of the 579-bp *tk* gene. One of the primers also contains a heptadecanucleotide of -21M13 (universal primer) at the 5' end. The DNA sequences were analyzed with a fluorescence-based DNA automatic sequencer after amplification of template with polymerase-chain-reaction (PCR). Among 14 mutations sequenced to date, 13 were C-to-T transitions. One C-to-A transversion mutation was also observed. Analyses of the DNA sequence around each mutated site suggest that the mispairing of thymine with guanine in the template is enhanced when the next nucleotide to be incorporated is dCTP or dTTP, while suppressed when the next nucleotide is dGTP. The 5' neighbor nucleotide of the mismatch may influence mutation frequency as well. Present observations with the forward mutation assay here are consistent with previous results from an *rII* reversion assay, supporting our model that the mutator phenotype displayed by *ts* LB3 is a consequence of perturbation of dNTP supplies to replication sites due to partial impairment of thermolabile deoxycytidylate hydroxymethylase at a semi-permissive

temperature.

The implication of this fast forward mutation approach is that mutagenic specificity induced by any mutagenic conditions, including imbalanced dNTP pools and chemical or physical mutagens, can be readily studied quantitatively and qualitatively at the DNA sequence level.

Key words: forward mutation; replication fidelity; deoxyribonucleotide pools; thymidine kinase; dCMP hydroxymethylase; T4 bacteriophage

2. Introduction

T4 dCMP hydroxymethylase, encoded by gene 42, is an essential viral protein, which converts dCMP to 5-hydroxymethyldeoxycytidine monophosphate, which is further phosphorylated by kinases for viral DNA replication (Mathews and Allen, 1983). Some years ago, temperature-sensitive gene 42 mutations have been shown to be lethal at high temperature (42° C) and mutagenic at semipermissive temperature (34° C) (Chiu and Greenberg 1973, Drake 1973), although the molecular basis of mutation stimulated by these mutants is still not clear (Williams and Drake 1977, Chiu et al 1977, Thylén and Mathews 1989, Wang 1990, Ji and Mathews 1991). Using T4 *rII* genes as genetic markers, thought to revert to wild type by defined mutation pathways, Williams and Drake (1977) characterized the mutator phenotype for some temperature-sensitive gene 42 mutants on the basis of responses to different mutagenesis. By this indirect criterion, they found that the principal mutation pathway stimulated by these mutations at semipermissive temperature is GC-to-AT transition. Recently, we have directly sequenced reversion mutations stimulated by one *ts* gene 42 mutant, *ts* LB3 and found that both GC-to-AT transition and GC-to-TA transversion mutations are stimulated simultaneously within one *rII* marker (Ji and Mathews 1991). The observed mutation pathways in the *rII* reversion system could be explained as mutagenic effects of *ts* LB3 and/or as specific genetic marker-associated events. Such effects of DNA context on type and frequency of mutations have been demonstrated in vitro and in vivo (Fersht 1979, Petruska and Goodman 1985, Kunkel 1988, Meuth 1989). By using different *rII* genetic markers, revertable to wild type

along GC-to-AT transition pathway, we found that mutation frequencies stimulated by *ts* LB3 differ among four genetic markers by as much as ten-fold, suggesting that local nucleotide context contributes to the *ts* LB3 mutator phenotype.

For further investigation and understanding of how DNA context influences mutation frequency as well as mutation type, a forward mutation assay, rather than a reversion assay, is required. By comparing different nucleotide contexts around each mutation site in a forward mutation assay system, one can precisely study DNA site specificity as well as mutagenic specificity induced by a mutagenic treatment.

In this study, we developed a forward mutation assay with the T4 thymidine kinase gene as a marker, and we used that system to further characterize the *ts* LB3 mutator phenotype. This system can readily be adapted for analysis of any mutagenic process in T4 bacteriophage.

3. Methods and materials

Reagents

Reagents for fluorescence-based sequencing reactions were from the Applied Biosystems Inc. (ABI) "automatic DNA sequencing reagent kit", including fluorescence-labeled universal primers -21M13 (dye primer), deoxyribonucleoside triphophates (dNTPs), and dideoxyribonucleoside triphosphates (ddNTPs). Other nucleotides, nucleosides and base analogs were from Sigma or Calbiochem. [^{3}H]-thymidine was from NEN. *Taq DNA* polymerase for polymerase-chain-reaction was from Promega or ABI.

Primers for amplification of T4 thymidine kinase gene were synthesized on a 380B DNA synthesizer from ABI by Dr. R. McParland of the Center for Gene Research and Biotechnology, Oregon State University.

Media

Nutrient broth had 8 g of Difco nutrient broth and 5 g NaCl per liter; nutrient agar plates had 4 g nutrient broth, 5 g NaCl and 23 g of Difco nutrient agar per liter.

Synthetic medium contained 5.8 g NaCl, 3.7 g KCl, 0.11 g $CaCl_2$, 0.10 g $MgCl_2$-$6H_2O$, 1.1 g NH_4Cl, 0.27 mg $FeCl_3$-$6H_2O$, 12.2 g tris (hydroxymethyl) aminomethane, 0.14 g Na_2SO_4, 0.1 g glycerophosphoric acid disodium salt, 5 g glucose and 1 g vitamin-free Casamino acids per liter. Medium pH was adjusted to 7.4 with HCl. Synthetic agar for plates contained synthetic medium with 20 mg/ml L-tryptophan and Bacto-agar, 12 g per liter in the bottom layer and 7 g

per liter in the top layer (Goscin and Hall 1972).

Bacterial and phage strains

E. coli KY895, an isoleucine- and valine-requiring strain which also lacks thymidine kinase activity, isolated from *E. coli* W3110 (F^-, K12 strain) by Igarashi et al (1967) is from D. Hruby, Dept. of Microbiology, Oregon State University. These bacteria were grown in synthetic medium containing 5 μg/ml thiamine-hydrochloride and plated on synthetic medium containing 2 μg/ml of the same chemical. *E. coli* B (wild type) and T4 phage strains, T4D (wild-type) and T4 *ts* LB3 (mutant) were from this laboratory. T4 *ts* LB3 is a temperature-sensitive gene 42 mutant which specifies a thermolabile dCMP hydroxymethylase.

Selection of *tk*$^-$ mutants

Overnight cultures of *E. coli* B grown in nutrient broth were diluted 1:100 into nutrient broth and grown with aeration at 34^o C to 2 X 10^8 cells per ml. Immediately before infection, 20 μg/ml L-tryptophan was added to each cell culture. T4 *ts* LB3 phage were used to infect *E. coli* B, with a multiplicity of 0.1 phage per cell. Infections were terminated 4 hours after incubation at 34^o C, by adding a few drops of chloroform to the infected cultures. The lysate was diluted with synthetic medium and plated on *E. coli* KY895 for selection of thymidine kinase-defective mutants, as described by Chace and Hall (1973). About 2 X 10^8 fresh *E. coli* KY895 cells were added with phage to synthetic top agar (2.5 ml), containing the following additions: 50 μg

thiamine-hydrochloride , 5 μg BrdUrd, 100 μg FdUrd, 500 μg uridine and 100 μg dAdo. The plates were allowed to stand at room temperature (26^{o} C) overnight under a 5-Watt fluorescent desk lamp at a distance of 11 cm from the plate. Plaques selected from bromodeoxyuridine-containing agar were further identified by growing again in BrdUrd-containing medium, followed by assay for thymidine kinase activity in phage-infected cells.

Single plaques selected from BrdUrd-containing agar plates were used to infect *E. coli* B, and each cell lysate was stored at 4^{o} C as the *tk*$^{-}$ phage stock for PCR. Phage particles in each lysate were directly used for template amplification and DNA sequencing with polymerase-chain-reaction, without further purification of phage DNA.

Preparation of extracts for enzyme assays

Fresh *E. coli* KY895 was grown in nutrient broth at room temperature (26^{o} C) to a concentration of 2.5 X 10^{8} cells/ml. Tryptophan (20 μg/ml) was added to the cells, and immediately afterward phage were added. The infection was stopped by rapidly chilling the cells on ice, and the cells were centrifuged 5 minutes at 6000 X g and resuspended in 0.5 M Tris-HCl buffer, pH 7.8. The resuspension was subjected to sonic oscillation 3 times for 20 seconds each, with intermediate cooling on ice. The supernatant fraction of each crude extract was used for enzyme assays after centrifugation at 12,000 X g for 10 minutes.

Thymidine kinase assay

5.0 µl of enzyme extract was mixed with 3.0 µl of *tk* assay solution containing 170 mM NaH_2PO_4 (pH 6.0), 17 mM ATP, 17 mM magnesium acetate and 100 µCi of [^{3}H]-thymidine. After the reaction mixture was incubated at 30^0 C for 45 minutes, 40 µl of ice cold double distilled water was added and the mixture placed in a heat block at 100^0 C for 3 minutes. 40 µl of supernatant was pipetted onto labelled DE-81 filters, each of which was washed immediately 3 times with 4 mM NH_4-formate/10 µM thymidine for 5 minutes each, twice with double distilled water for 5 minutes each and 2 times with 95% ethanol for 5 minutes each. Each filter was dried by air and counted for radioactivity in a scintillation vial.

Gene amplification

Four primers were designed and synthesized on a DNA synthesizer, based on the known T4 thymidine kinase sequence (Valerie et al 1986). 1) primer tk1 (CTA TCG ATA AAG CTG AAA ATG) is located 39 to 59 bp upstream of the translation start of the *tk* gene in the sense strand. 2) primer tk2 (CCC CTT TAG TTA GAT AAA CC) is located 18 to 37 bp downstream of the translation end of the *tk* gene in the antisense strand. 3) primer -21tk1 (TGT AAA ACG ACG GCC AGT CTA TCG ATA AAG CTG AAA ATG) is a primer of tk1 coupled with -21M13 sequence (17mer). 4) primer -21tk2 (TGT AAA ACG ACG GCC AGT CCC CTT TAG TTA GAT AAA CC) is a primer of tk2 coupled with -21M13 sequence. The thymidine kinase gene was amplified with either combination of tk1 with -21tk2, or tk2 with -21 tk1 as primers

for polymerase chain reaction in a thermal cycler form Ericcomp Inc..

The PCR reaction mixture (50 or 100 µl) contains 10 mM Tris chloride (pH 8.0), 50 mM KCl, 1.5 mM $MgCl_2$, 25 µM each dNTP and 5 pmoles each primer, with 10^4 phage particles as template. Immediately before running PCR, 2 units of *Taq* polymerase was added, and the PCR solution was covered with 50 µl of mineral oil to prevent evaporation. The PCR thermal cycle is as follows, with 30 cycles: 96° C for 30 sec, 50° C for 1 minute and 72° C for 2 minutes. The PCR products were identified by electrophoresis on a 1.2% agarose gel.

DNA sequencing

Sequencing reaction mixtures were prepared as follows. Each A or T reaction mixture contained 2.0 µl of dye primer (0.4 pmoles/µl), 2.0 µl of 5X sequence buffer, 2.0 µl of dNTPs/ddNTP mix, 1.5 µl of DNA template direct from PCR amplification mixture, and 1 µl of *Taq DNA* polymerase (5 units/µl). The G or C sequencing reaction mixtures contained twice as much reaction mixture as that of A or T. The sequencing reaction is programmed with two different cycles with 10 times each. Program 1 included three steps at 95° C for 30 seconds, 60° C for 1 second and 70° C for 1 minute and program 2 at 95° C for 30 seconds and 70° C for 1 minute. The four sequencing reaction mixtures, A, T, G, and C were pipetted together with 300 µl of 95% ethanol and 9 µl of 3 M sodium acetate (pH 5.3) and incubated on ice for 15 minutes. DNA was precipitated after centrifugation at 12,000 X g for 20 minutes. The pellets were dissolved in 6 µl of deionized formamide/50 mM EDTA (pH 8.0). The samples were incubated at 90° C water bath for 2 minutes, and loaded immediately onto a pre-warmed 6% polyacrylamide gel on an ABI 370A DNA sequencer (Gibb et al 1989).

4. Results and discussion

Rationale for this study

As noted earlier, the nucleotide sequence context for mutagenesis is best understood through a forward mutation assay system. Such systems are potentially targeted toward the *rII*, *e* , *td* and *frd* genes in T4 bacteriophage (Drake and Ripley 1983), because these are all relatively small genes, where mutation can be detected by specific plaque morphology tests. However, we learned that none of these are practically useful for analyzing mutation events at the DNA level. The most difficult aspect is selection of mutations from wild type populations based on different plaque morphology between wild-type and mutations. Assuming that mutation frequency stimulated by a mutator, such as *ts* LB3, in these genes, is 10^3-fold higher than the spontaneous mutation rate (10^{-7} mutation/per targeting gene), we would still have to plate out ten thousand phage in order to pick up one mutation. Several dozen independently isolated mutants must be analyzed in order to obtain a meaningful mutation spectrum. However, targeting at the thymidine kinase (*tk*) gene seemed attractive, because the *tk*-defective mutants can be selected and identified easily in BrdUrd-containing medium. After BrdUrd incorporation into DNA, only *tk*-negative mutants can survive, while wild-type phage are killed under near-ultraviolet light (Chace and Hall 1973).

Further facilitation of this forward mutation approach comes from recent developments in DNA amplification and sequencing techniques. With polymerase-chain reaction, a target gene bearing a specific mutation can be amplified for DNA sequencing without cloning (Mullis and Faloona 1987, Saiki et al 1988). Moreover, by using fluorescence-

labelled primers, amplified DNA can be directly sequenced with an automatic DNA sequencer, such as the ABI model 370A DNA sequence system (Smith et al 1986, Gibb et al 1989). The principle in the DNA sequencer is the same as that of the Sanger dideoxy method (Sanger et al 1977), except for the use of a different detection approach for synthetic DNA fragrments. There are four fluorescence-labeled primers, one distinctive color corresponding to each dideoxynucleotide during DNA sequencing. DNA sequence is detected as each DNA fragment migrates past a laser which excites the fluorescence-labelled primer. The advantage of using the ABI model 370A DNA sequencer comes with the large capacity of the instrument: up to 12 samples can be sequenced on each gel in 12 hours, with 350 to 500 bases of reliable DNA sequence for each template.

Commercially available primers include universal primer (-21M13), reverse universal primer, T7 and others. In principle, this automatic sequencing approach is applicable only for those DNA templates that contain the complementary sequence for fluorescence-labelled primers. Although it is hard to synthesize specific fluorescence-labelled primers for each individual target gene (Smith et al 1986), commercially available fluorescence primers can be used for sequencing with the following modification. If -21M13 sequence is coupled with a specific target gene primer (*tk* gene in this study), the amplified template after PCR with this primer can be sequenced by this machine, as shown in Figure IV-1.

Mutant selection

Studies in this laboratory and by others have shown that infection of a host by *ts* LB3 at 34° C is a mutagenic condition (Williams and Drake 1977, Ji and Mathews 1991). Therefore, 34° C was used for stimulation of mutagenesis in this study. After infection of *E. coli* B with *ts* LB3 at 34° C, mutations at the thymidine kinase locus, induced by thermolabile dCMP hydroxymethylase, were selected on BrdUrd-containing agar plates. The procedure is fast and reliable. The *tk*$^-$ phage plaque can form in 6 to 12 hours at room temperature on the plate under light. The *tk*$^-$ phage phenotype was further identified by infecting host cells again in BrdUrd-containing medium and by assay of thymidine kinase activity in phage-infected cells. All phage lysates grown from plaques selected from the plate can grow in BrdUrd-containing medium and all tested have no detectable thymidine kinase activity, as shown in Figure IV-2. Since the wild-type host, *E. coli* B, has high activity of thymidine kinase, it is necessary to use a *tk*$^-$ host strain, both for selection of *tk*$^-$ phage in BrdUrd-containing medium and confirmation of phage phenotype with enzyme assay.

In order for our sequence studies to truly analyze independent mutational events induced by partial inhibition of a thermolabile dCMP hydroxymethylase, two questions must be addressed. 1) What is the likelihood that any mutation analyzed might have arisen spontaneously? As we reported earlier (Ji and Mathews 1991), by using the *rII* reversion mutation assay, we found 8- to 80-fold stimulations of GC-to-AT reversion mutations at 34° C. If we use 20-fold as an average, then 196 G-C base pairs in the *tk* coding region should undergo induced mutations about 4,000-fold more rapidly than spontaneous background mutations (10^{-9} mutation/base-pair). Therefore, it seems unlikely that

the selected mutations are spontaneous mutations.

2) Are the different mutants analyzed of independent origin? Since there are about 8 rounds of viral DNA replications in one cycle of infection (Mathews and Allen 1983), and about 2 x 10^7 phages were used to infected 2 X 10^8 cells of *E. coli* B, we expect to observe about 2,500 independent mutational events among a total of 7,000 mutations during first phage growth circle as listed in Table IV-1. Therefore, the possibility of picking up 2 mutants reverting from the same original mutational event in a dozen samples, should be less than 13.0% after first phage growth cycle. Since only 10% of the cells are infected in the first phage growth cycle, additional phage growth cycles should accumulate more independent mutation evens and reduce the chance for picking up mutations with the same mutational origin.

Template amplification

Phage particles in the lysate were used directly for template amplification with polymerase-chain-reaction, without further purification of phage DNA, in contrast to our precedures in previous studies (Ji and Mathews 1991). With the PCR protocol used here, the efficiency of direct amplification of template with phage lysate is the same as that obtained by using phage DNA. Using one specific primer and one nonspecific primer that contained a specific heptadeca-nucleotide (-21M13, universal primer) on the 5' end, we amplified the *tk* gene. The PCR-products about 700 bp in length, were found on the agarose gels as shown in Figure IV-3. With this new protocol, the efficiencies of amplification are even better than those using conventional protocols (Ji and Mathews 1991).

Automatic sequencing with fluorescence-labelled primer

Since we used lower concentrations of dNTPs and primers in PCR mixtures than in our earlier study (Ji and Mathews 1991), the PCR products were used directly for sequencing without further removing dNTP and primers. Using fluorescence-labelled primer -21M13, sequencing products were loaded into 6% polyacrylamide gels and analyzed by the ABI 370A DNA sequencer as shown in Figure IV-4. DNA Sequence obtained from the crude PCR-amplified template is reliable the same as that obtained from the purified single-strand M13 template.

We also learned that using 10^4 phage particles in each lysate as initial templates for amplification gives the best results for both PCR amplification and automatic DNA sequencing. Fewer than 10^2 phage particles as PCR template decreased PCR efficiency, while more than 10^6 phage particles decreased sensitivity of DNA sequencing. This is because DNA automatic sequencing is based on the amount of amplified template which contains the -21M13 complementary sequence.

Mutagenic specificity induced by *ts* LB3 at 34° C

We have sequenced the *tk* gene of 14 thymidine kinase-negative mutants induced by ts LB3 at semipermissive temperature. All mutants sequenced contain point mutations, as shown in Table IV-2. Thirteen of fourteen mutations sequenced are GC-to-AT transitions, while one is a GC-to-TA transversion. All of these mutations changed a sense codon either to another amino acid or to a stop codon. These results are consistent with early observations with the *rII* reversion assay, supporting the conclusion that the principal mutator phenotype of *ts*

LB3 is GC-to-AT transition during infections at semipermissive temperature (Williams and Drake 1977, Ji and Mathews 1990). Comparing our sequence data with those reported by Valerie et al (1986), we found one sequence discrepancy, at position # 62 from *tk* translation start site with C instead of T. However, this correction of DNA sequence did not change the protein sequence, since both codons (CTG) and (TTG) code for leucine.

Likely mutation pathways for misincorporation and mispairing stimulated by *ts* LB3 at semipermissive temperature during DNA replication are as proposed in Figure IV-5. We postulate that depletion of hm-dCTP around replication sites, due to partial inhibition of thermolabile dCMP hydroxymethylase at semipermissive temperature, stimulates dTTP competition with hm-dCTP and mispairing with guanine in the template, causing T-to-C transitions. The C-to-A transversion could occur through competition between dATP and depleted hm-dCTP, and mispairing of dAMP with guanine in the template.

DNA site specificity induced by *ts* LB3 at 34° C

Using different *rII* markers in our previous studies, we found about ten-fold higher mutation frequency with marker *rII* SN103 than that with other GC-to-AT transition genetic markers. Through sequencing the *rII* SN103 marker, we identified the CC cluster as a likely sequence context contributing to high mutation frequency. By using the forward mutation assay here, we compared the different dinucleotide frequencies in the *tk* coding region with observed frequency at mutation sites as listed in Table IV-3. Based on the limited number of mutations analyzed to date, we found that both C or T as

nearest nucleotide at either 5' or 3' site correlated with high mutations, while no A at 5' mutation site and G at 3' mutation site were observed in *tk*$^-$ mutants.

Are these neighbor nucleotides determinants for DNA site specificity of mutagenesis stimulated by *ts* LB3 at 34^0 C? Although an answer to this question is uncertain yet, due to limited information about mutation sequences to date, the observed nucleotide context associated with high mutation rate is consistent with our current understanding of mutagenesis. Fersht and others have found that imbalanced dNTP pools can interfere with proofreading efficiency (Fersht 1979, Meuth 1989, Phear and Meuth 1989). The next nucleotide in a DNA template strand can enhance transition errors by "pushing" the polymerization complex past the mismatch when the next nucleotide to be incorporated is present in excess, thereby diminishing the effectiveness of the 3'-5' proofreading exonuclease. Since both our previous study (Ji and Mathews 1991) and present observations here (Table IV-1 and Figure IV-5) showed that the most highly imbalanced dNTP pool ratio induced by *ts* LB3 is dTTP/hm-dCTP at replication sites, the observed high mutations at CT dinucleotide sites are in good agreement with the next nucleotide model. In fact, from the thermodynamic point of view, as discussed below, T as nearest nucleotide at mutation site should be much more unfavored.

Apparently, it seems unlikely that only the nearest nucleotide influences proofreading activity. With studies of T4 DNA polymerase in vitro, Sinha (1987) found that the error can be excised as many as four normal base pairs downstream from an error before editing can no longer be detected, suggesting that at least four nucleotides downstream from a mismatch can influence proofreading efficiency. By studying 2-

aminopurine mutagenesis in vitro, Goodman and his coworkers proposed that base stacking energies around mutation sites are critical for mispairing (Petruska and Goodman 1985, Goodman 1988). The relatively stable regions of DNA might be proofread with greater difficulty than those located in less stable regions. The stability of base pairs present at the upstream 5'-side of a misinsertion site can affect proofreading by increasing the probability that polymerase cycles to a melted out DNA configuration following misinsertion. The stability of base pairs present at the "downstream" 3'-side of a misinsertion site can affect proofreading by modulating the ability of the exonuclease to "peelback" correct base pairs to confront an error that escaped initial proofreading. Our vivo data support this notion. Comparing DNA context at mutation sites, we found a high correlation between base stacking energy and mutation frequency. However, before we draw any definite conclusion about effects of DNA sequence context on mutation specificity, further accumulation of sequence information around mutation sites stimulated by various dNTP pool perturbations is necessary. Using this forward mutation approach, further investigation of mutagenic specificity and DNA site specificity is under way.

Acknowledgement

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Table IV-1. Mutations stimulated by *ts* LB3 during the first phage growth cycle[1]

Replication cycle	Independent mutations	Progeny per mutation	Total mutation
1	20	50	1000
2	40	25	1000
3	80	12.5	1000
4	160	6.25	1000
5	320	3.125	1000
6	640	1.5625	1000
7	1280	0.78125	1000
Total	2560	100	7000

[1]The assumption used in this caculation is that the mutation rate per phage growth cycle is equal throughout several rounds of replication. Since the mutation rate in *ts* LB3 is about tenfold higher than the spontaneous mutation rate (about 10^{-8}/bp), there are 200 GC mutation targets in the *tk* gene, and about half of the mutations are selectable, the mutations stimulated by *ts* LB3 with 2 X 10^7 phages in the first round of replication, which are fixed in the second round of replication, should be 2 X 10^7 phage X 10^{-8} mutation rate/base-pair X 200 GC base-pair X 50% selectable mutation = 20 independent mutations. Therefore, the possibility of picking up 2 mutants reverting from the same original mutational event in a dozen samples after first phage growth cycle, should be C_{12} [20 X (50/7000 X 49/7000) + 40 X (25/7000 + 24/7000) + 80 X (12.5/7000 X 11.5/7000) + ...] = 12.7%

Table IV-2. Mutations in *tk* locus stimulated by *ts* LB3 at 34° C[1]

Observed mutations	Position[2] of mutation	Base substitution	Mutation[3, 4] sequence	Amino acid change(s)
4	26	C-to-T	TATG T AGCA	Ala-to-Val
4	47	C-to-T	AAAT T TGCT	Ser-to-Phe
1	248	C-to-A	TACG A AATG	Cys-to-Phe
1	281	C-to-T	AGCT T AGTT	Stop
1	343	C-to-T	TAAG T CATA	Gly-to-Ser
2	350	C-to-T	TAGC T CATA	Gly-to-Glu
1	532	C-to-T	GTTT T TTTG	Ser-to-Phe

[1] One sequence discrepancy in *tk* gene reported by Valerie et al (1986) is located at #62 with a C rather than a T.

[2] The nucleotide numbering is from *tk* translation start.

[3] Mutations could occur on either tk strands. Mutations on the antisense strand are underlined. The strand is shown from 5' to 3'.

[4] The middle nucleotide in the sequence is a mutation site, which is mutated from wild-type "C" to "T" or "A" as shown.

Table IV-3. Dinucleotide frequencies inside *tk* coding sequence and at target sites of *ts* Lb3-induced mutations

Target[1] dinucleotide	Frequency[2] in *tk*	Frequency at[3] mutation site	Ratio of[4] Frequency
-**C**T-	.335	.3846 (5)	1.15
-**C**A-	.391	.3846 (5)	0.98
-**C**C-	.132	.2307 (3)	1.75
-**C**G-	.142	.0000 (0)	0.00
-T**C**-	.324	.4615 (6)	1.42
-G**C**-	.257	.3846 (5)	1.50
-C**C**-	.124	.1538 (2)	1.24
-A**C**-	.295	.0000 (0)	0.00

[1] The nucleotide altered by the substitution (to C) is boldfaced. Only transition mutations are analyzed here, which represent 13 of 14 mutational events.

[2] Proportion of the given dinucleotide of all CN or NC dinucleotides at target sites.

[3] Proportion of the given dinucleotides at target sites. Numbers in parentheses represent the number of mutants with given target sequence.

[4] Ratio of frequency at mutation site with whole *tk* gene.

Figure IV-1. Schematic steps for DNA sequencing with dye primer

A). The region of genomic DNA to be amplified is indicated by the open rectangles. Two strands with their 5' to 3' orientation are shown. The darkened regions represent flanking sequences.

B). The oligonucleotides anneal to sites just outside the sequence to be amplified. One of the oligonucleotides has a -21M13 universal primer sequence as shown with "✽——".

C). PCR consists of repetitive cycles of denaturation, annealing with primers, and DNA polymerization. Final PCR products are amplified target DNA segments with defined ends after 30 cycles.

D). Since about half of the amplified strands contain -21M13 complementary sequence as shown with "*——", dye primer -21M13 (fluorescence-labelled primer) as shown "⊛——"(asterisk with circle) can be annealed with amplified DNA for sequencing reaction.

E). Dideoxy DNA sequencing data are obtained.

Figure IV-1

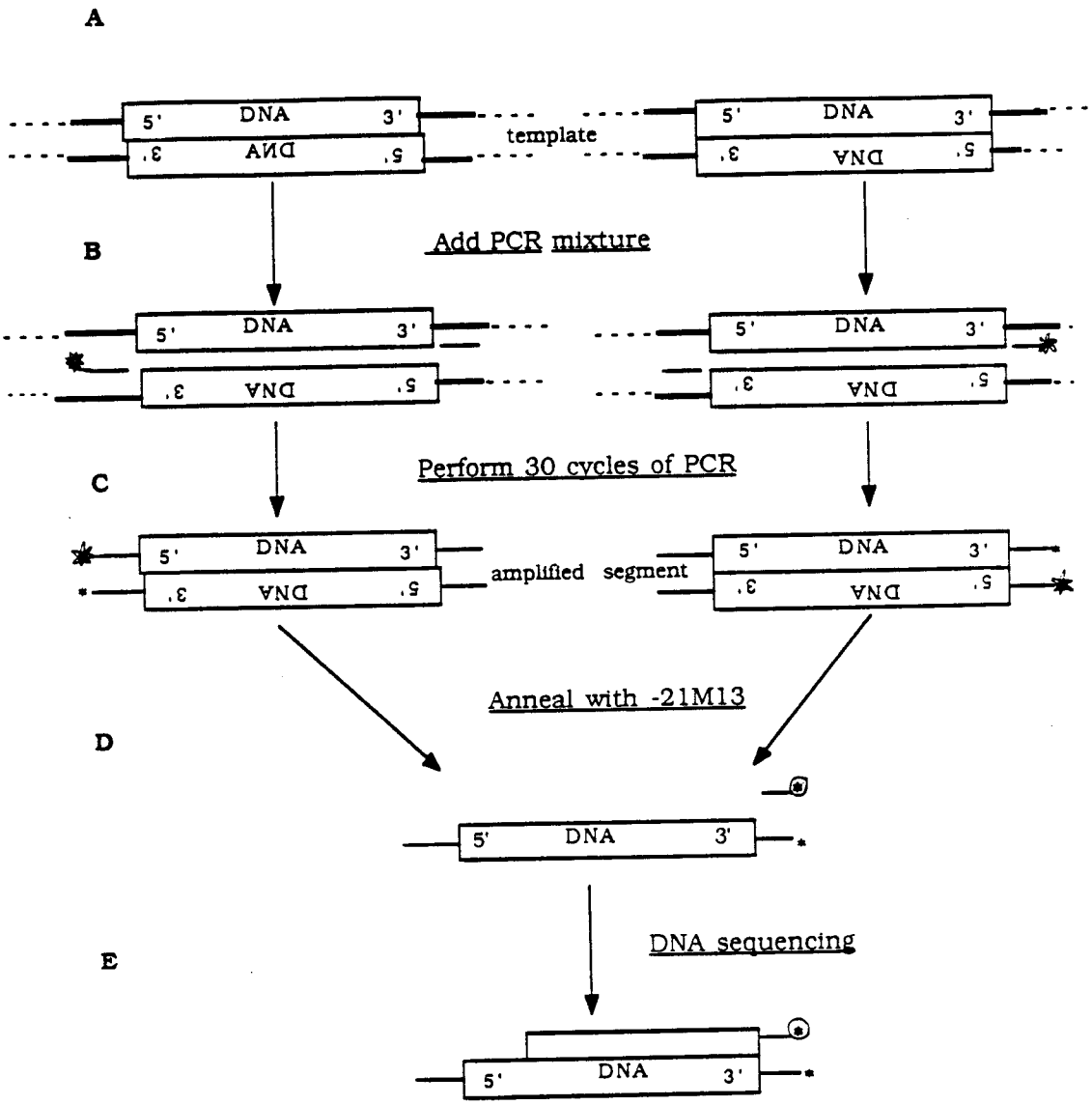

A
5' DNA 3'
3' DNA 5'
template
B
Add PCR mixture
C
Perform 30 cycles of PCR
amplified segment
D
Anneal with -21M13
E
DNA sequencing

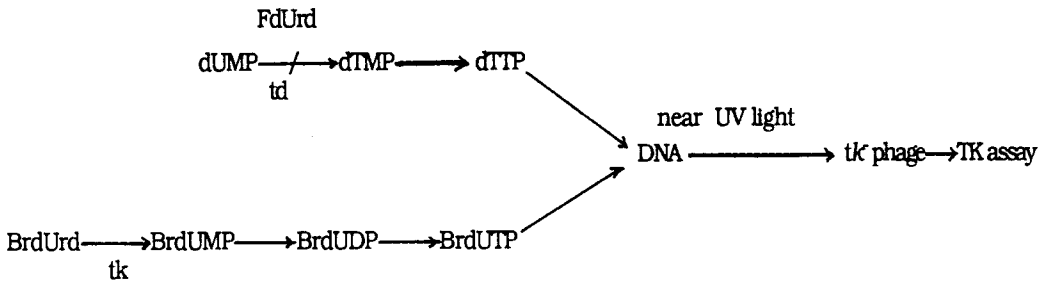
Figure IV-2. Selection of *tk*$^-$ phage and assay of enzyme activity

A. Selection of *tk*$^-$ phage. Selection medium contained FdUrd for inhibiting thymidylate synthase activity and ensuring BrdUTP incorporation into *tk*$^+$ phage DNA; and dAdo for preventing fluorouracil incorporation into mRNA. BrdUrd-containing DNA in *tk*$^+$ phage were broken down under near-ultraviolet light.

B. Thymidine kinase activity assay. Crude extracts of phage-infected cells or host cells were used for thymidine kinase activity assay. The enzyme activity in T4D-infected cells was designated 100%. The data plotted here are average values from three repeats; these agreed within 10% variation.

Figure IV-2

A



B

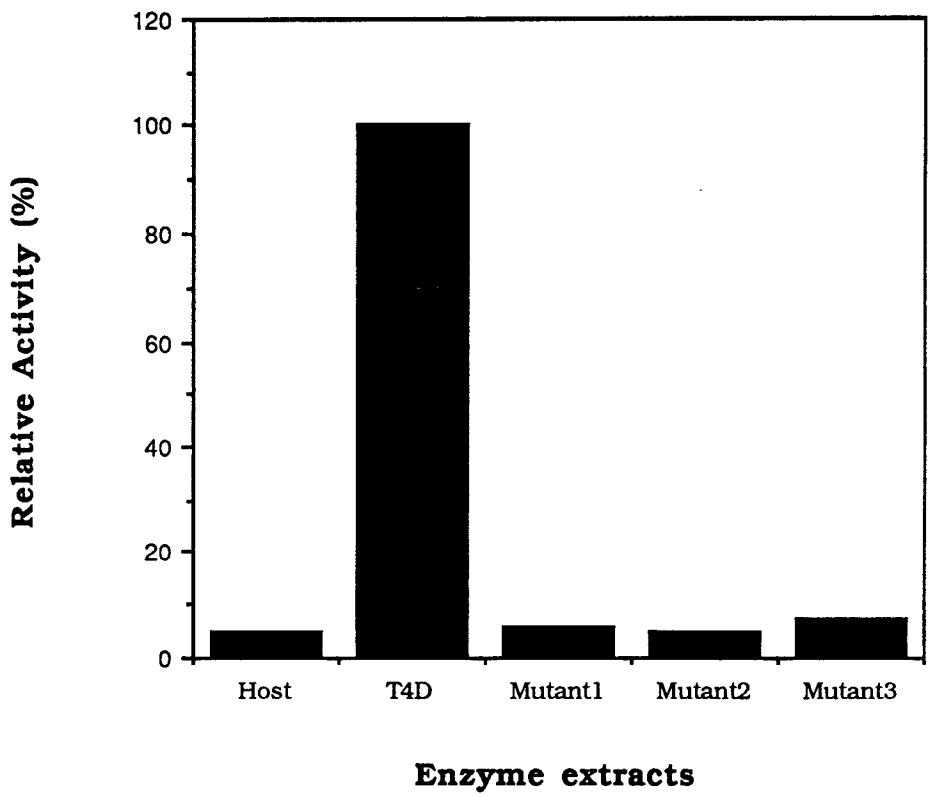


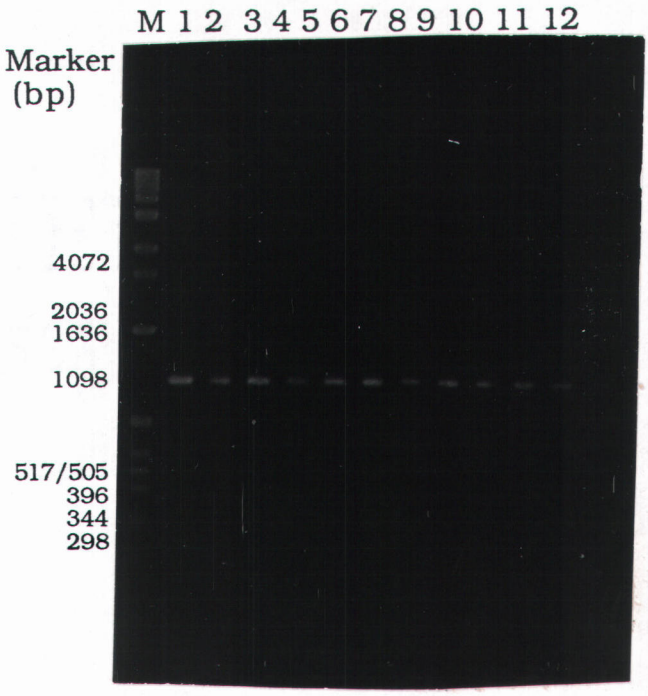
Figure IV-3. Template amplification with PCR

A. PCR-amplified *tk* gene. The *tk* gene in each of 6 mutants was amplified and analyzed in this 1.2% agarose gel. Lanes 1-6: amplified with primers tk1 and -21tk2. Lanes 7-12: amplified with primers tk2 and -21tk1.

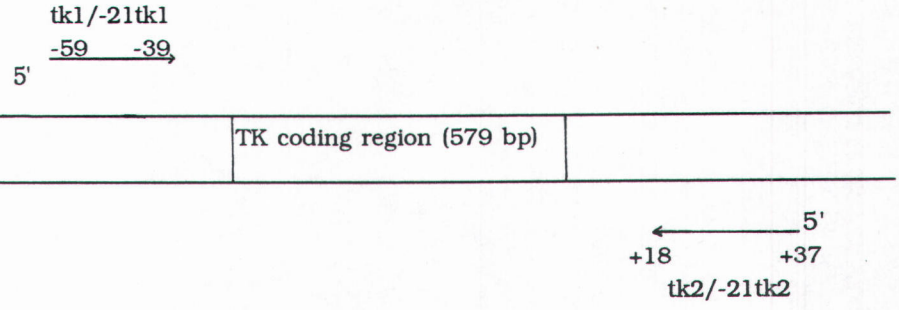
B. Primers and their locations around the *tk* gene. Primer tk1 is located 39 to 59 bp upstream of the tk coding region in the sense strand; Primer tk2 is located 18 to 37 bp downstream of the *tk* coding region in the antisense strand. Primers -21tk1 and -21tk2 are the primers with the 17mer -21M13 sequence at the 5' ends of primer tk1 and tk2, respectively.

Figure IV-3

A



B



1)tk1

CTA TCG ATA AAG CTG AAA ATG

2)tk2

CCC CTT TAG TTA GAT AAA CC

3) -21tk1

TGT AAA ACG ACG GCC AGT CTA TCG ATA AAG CTG AAA ATG

4)-21tk2

TGT AAA ACG ACG GCC AGT CCC CTT TAG TTA GAT AAA CC

Figure IV-4. DNA sequencing with fluorescence-labelled primer

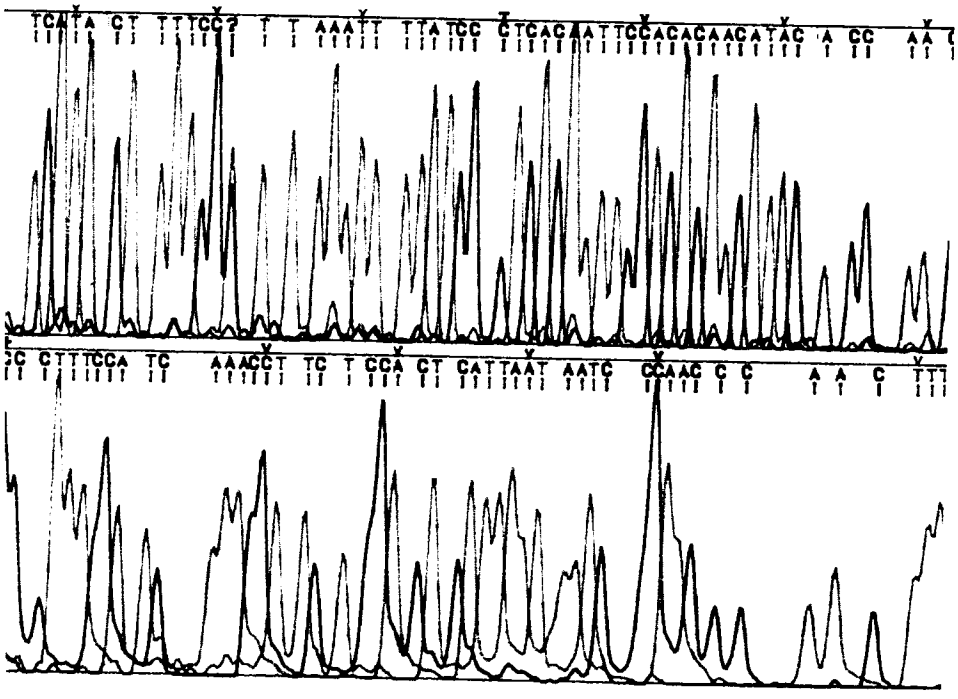
Fluorescence-labelled -21M13 "dye" primers were used for sequencing. Four sets of sequencing reactions, A, T, G, C were combined and loaded upon one lane on 6% polyacrylamide gels. Four dyes in sequence are green for A, red for T, yellow for G and blue for C, respectively.

A. Control; purified M13 single strand DNA as templates

B. *tk* sequence; crude PCR-amplified DNA as templates

Figure IV-4

A



B

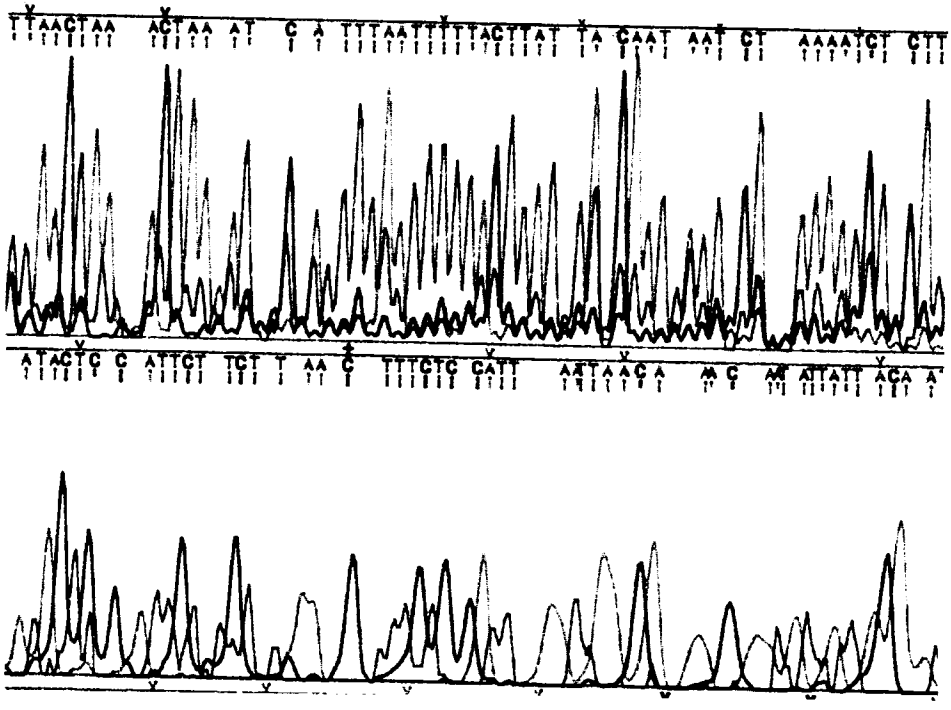
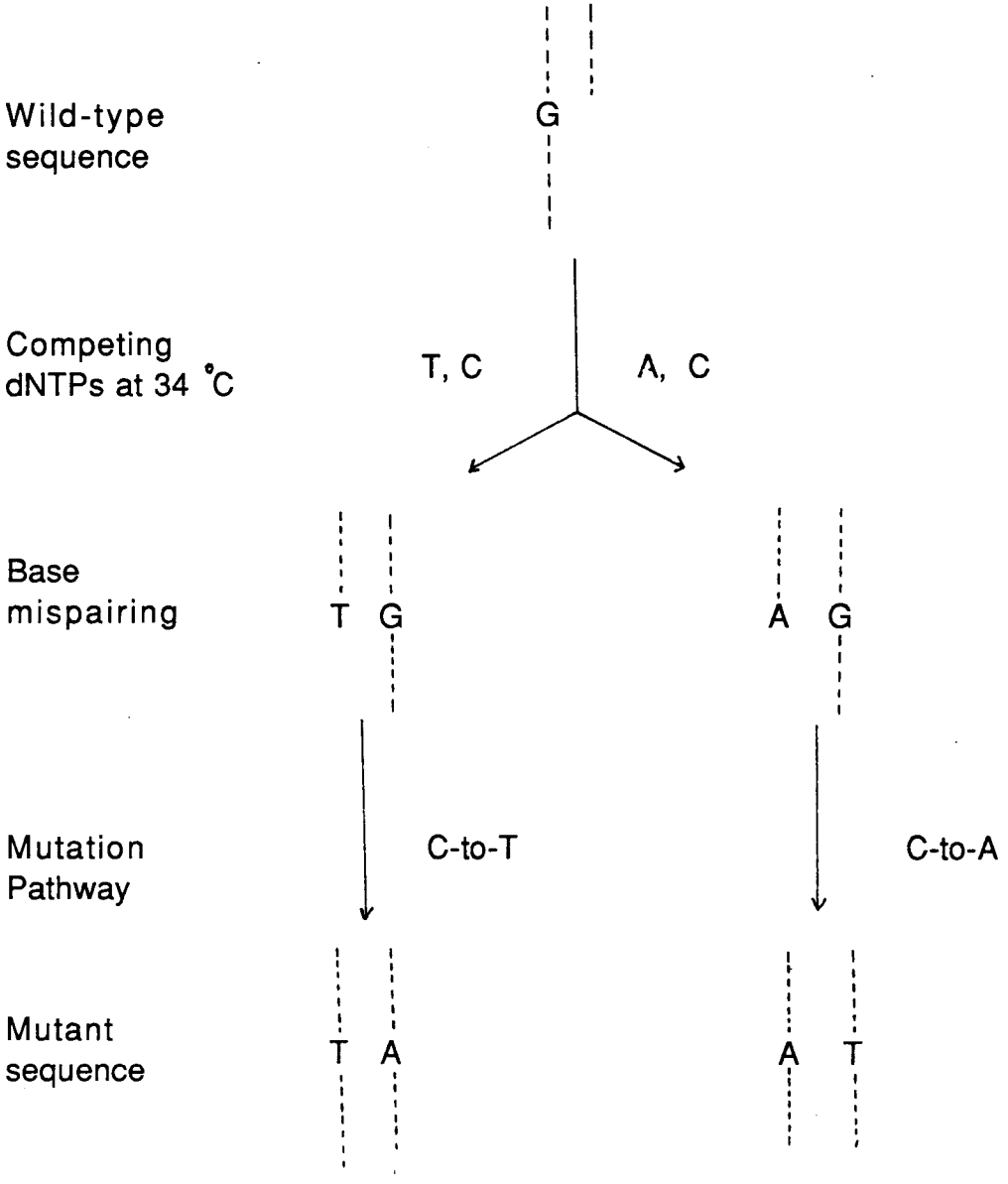


Figure IV-5. Proposed mutation pathways stimulated by *ts* LB3 at 34° C

C-to-T transition is proposed to occur when dTTP competes with hm-dCTP to mispair with guanine in the template, due to depletion of hm-dCTP pool at replication sites, which is caused by thermolabile dCMP hydroxymethylase at semipermissive temperature. C-to-A transition also may occur when dATP competes with hm-dCTP to form an A-G mispair, although this mutation pathway is apparently minor.

Arrows "⟶ " indicate rounds of replication. Long lines "----N----" represent DNA template strands and short lines "----N " represent new synthesizing strands.

Figure IV-5


Wild-type sequence
G
Competing dNTPs at 34 °C
T, C
A, C
Base mispairing
T G
A G
Mutation Pathway
C-to-T
C-to-A
Mutant sequence
T A
A T

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