AN ABSTRACT OF THE THESIS OF

Roy Geoffrey Sargent for the degree of Doctor of Philosophy in

Biochemistry and Biophysics presented on March 20, 1987.

Title: Deoxyribonucleotides as Determinants of DNA Replication Fidelity in Bacteriophage T4

Abstract approved: Redacted for privacy

Christopher K. Mathews

Imbalanced deoxyribonucleoside triphosphate (dNTP) pools are mutagenic for DNA synthesis in both intact cells and cell-free replication systems. Almost certainly, such mutagenesis involves competition between correctly and incorrectly base-paired precursors at replication sites. However, there are certain differences between the intact cell and cell-free systems that do not always allow direct comparisons to be made with regards to mutagenesis stimulated by dNTP pool imbalances. For example, even though dNTP concentrations can be carefully controlled *in vitro*, cell-free replication systems almost certainly do not contain all of the components found at replication forks *in vivo*. In contrast, intracellular dNTP concentrations, and base changes in DNA, have not always been measured in parallel with conditions thought to cause imbalanced dNTP pools and mutagenesis *in vivo*.

I describe in this dissertation the further development of a procaryotic system, namely bacteriophage T4, as a model *in vivo* system

for studying dNTP pool imbalances and mutagenesis. While these investigations focus on two enzymes in T4 deoxyribonucleotide metabolism, namely T4 ribonucleotide reductase and dCMP deaminase, the effects of other phage mutations on dNTP pools and mutagenesis were also investigated.

Loss of T4 dCMP deaminase activity during phage infections resulted in hyperexpanded hmdCTP pools, decreased dTTP pools, and expanded dGTP pools. There was a concomitant increase in AT-to-GC mutagenesis, which was confirmed by nucleic acid sequencing of amber$^+$ phage revertants. To some degree, the aberrant dNTP pools and AT-to-GC mutation rates could be returned to normal by the addition of thymidine to infections by the dCMP deaminase deletion mutant, pseTΔ4, but not during infections by the dCMP deaminase missense mutant, cdN16.

Both AT-to-GC and GC-to-AT mutations were weakly stimulated during T4 ribonucleotide reductase mutant, nrdBamB55, infections of ED8689/pPS2, a host cell overproducing Escherichia coli (E. coli) ribonucleotide reductase. During T4 nrd$^+$ infections, BrUdR mutagenesis preferentially stimulates GC-to-AT mutagenesis; while in uninfected E. coli, BrUdR stimulates the opposite pathway, namely AT-to-GC transitions. However, during nrdBamB55 infections of ED8689/pPS2, BrUdR mutagenesis now resembles that of uninfected E. coli, namely, AT-to-GC transitions prevail. I discuss a model based on these results that explains how BrUdR stimulates opposite mutagenic pathways in T4-infected and uninfected E. coli.

Deoxyribonucleotides as Determinants of DNA Replication Fidelity in Bacteriophage T4

by

Roy Geoffrey Sargent

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Completed March 20, 1987

Commencement June 1987

APPROVED:

Redacted for privacy

Professor and Chairman of Biochemistry and Biophysics

Redacted for privacy

Dean of Graduate School

Date thesis is presented March 20, 1987

Typed by Barbara Hanson for Roy Geoffrey Sargent

Acknowledgments

I want to thank Dr. Christopher K. Mathews for the opportunity to study and work in his laboratory these past few years. Dr. Mathews has not only been a mentor, providing encouragement, support, and independence for the pursuit of my research interests, but he has also co-participated in some unforgettable adventures.

I greatly appreciate the capable help provided by Meredith Howell with the RNA sequencing, and David Klock for running many of the dNTP assays.

My special thanks go to Dr. Mary Slabaugh and Dr. Jim Allen for friendship, understanding, and encouragement when they were needed.

I would like to gratefully acknowledge the receipt of Tartar fellowships the summers of 1985 and 1986.

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF TABLES

DEOXYRIBONUCLEOTIDES AS DETERMINANTS OF DNA REPLICATION FIDELITY IN BACTERIOPHAGE T4

I. INTRODUCTION

DNA replication results in as few as 1×10^{-8} to 1×10^{-12} errors per base pair per round of replication (Drake, 1970). In the absence of mutagenic agents during replication, this fidelity is the product of the accuracy by which the DNA template is copied, and the organism's ability to subsequently detect and repair mistakes. In turn, intracellular dNTP concentrations, the state of replication enzymes, and primary and secondary template structure all interact to determine local replication errors.

It has been well established that conditions promoting imbalanced deoxyribonucleotide pools can stimulate mutation frequencies in organisms actively replicating their DNA. It is less well established, however, as to first, how much change in intracellular dNTP pools is necessary to have a measurable effect on mutation rates. Second, in most cases the base changes in DNA have not been unambiguously identified, hence, obscuring the events leading to mutagenesis.

The goal of the research described herein, was to develop a system whereby I could manipulate and measure intracellular dNTP pools, and determine their influence on mutation rates during *in vivo* replication. Bacteriophage T4 offers several advantages in addressing these problems. First, T4 encodes most of the enzyme activities needed for deoxyribonucleotide metabolism during phage infection. The

Table I. Abbreviations

Term	Meaning
am	amber mutation
amp	ampicillin
β-ME	beta-mercaptoethanol
BrdUTP	5-bromodeoxyuridine triphosphate
BrUdR	5-bromodeoxyuridine
BSA	bovine serum albumin
cd	structural gene for dCMP deaminase
cdd	structural gene for dCTP deaminase
Δ	deletion mutation
dCMP Hmase	dCMP hydroxymethyltransferase
dCTPase	dCTP nucleotidohydrolase
ddNTP	2', 3'-dideoxyribonucleoside triphosphate
dNMP kinase	deoxyribonucleoside monophosphate kinase
DTT	dithiothreitol
dUTPase	dUTP nucleotidohydrolase
E. coli	*Escherichia coli*
EDTA	ethylene diamine tetraactic acid
EtOH	ethanol
gp	gene product
hmdCMP	5-hydroxymethyldeoxycytidylate
HPLC	high performance liquid chromatography
HU	hydroxyurea
IMP	inosinate
MeOH	methanol
NDP kinase	nucleoside diphosphate kinase
nrd	structural gene for ribonucleotide reductase subunit
ppi	pyrophosphate
rXTP	xanthosine ribonucleoside triphosphate
Serine hmase	serine hydroxymethyltransferase
SpeedVac	Savant Speed Vac Concentrator
TBE	tris-borate-EDTA
TCA	trichloroacetic acid
TdR	deoxythymidine
TEMED	N,N,N',N'-tetramethylethylenediamine
tet	tetracycline
TOA	tri-n-octylamine
Tris	(Tris-hydroxymethyl)aminoethane
ts	temperature sensitive mutation
UdR	deoxyuridine

structural genes for these T4-induced enzymes are identified, mapped on the T4 chromosome and several mutant alleles of each gene are available (Mathews and Allen, 1983). These mutant alleles offer a ready means to manipulate T4 deoxyribonucleotide metabolism and dNTP pools. Furthermore, the effects of dNTP pool imbalances on T4 DNA replication fidelity *in vitro* have been determined (Sinha and Goodman, 1983). Finally, there is an extensive library of well-defined mutations in the T4 *rII* genes that can be used to genetically identify mutagenic pathways in replicating phage. And, by dideoxy sequencing of *rII* messenger RNA, it has recently become possible to physically identify mutations in the *rII* genes.

A. General T4 Biology

There are several current reviews describing most of the general aspects of T4 molecular biology, including: Mathews et al., (1983); Rabussay and Geiduschek, (1977); and Mathews, (1971). Hence, this section summarizes various aspects of T4 molecular biology peripherally related to this dissertation, and attempts to convey a basic perspective of the organism and its life cycle.

Bacteriophage T4 is a large bacterial virus whose usual laboratory hosts are strains of *Escherichia coli* (*E. coli*) B and K. The T4 genome is a linear, double-stranded molecule of about 166 kilobase pairs (molecular weight = 1.53×10^8) in size, and has the capacity to encode over 100 proteins. This places T4, if classified by size, among the largest viruses yet discovered, along with the eucaryotic vaccinia and herpes viruses (Luria et al., 1978). About 90% of the T4

genome has been mapped, and roughly 50% of the known gene products are utilized as components of the T4 virion or as catalysts in its assembly (Mosig, 1983a). Most of the remaining gene products function in T4 DNA replication, deoxyribonucleotide metabolism and regulation of phage gene expression.

There is a characteristic sequence of events that can be used to describe the T4 life cycle. Infections are initiated by adsorption of phage to the host bacterium cell wall. A portion of the phage tail penetrates the cell wall and T4 DNA as well as some minor proteins are injected into the cell. Transcription of T4 genes by the host RNA polymerase essentially begins immediately and the first phage gene products appear within a few minutes.

T4 gene products can be classified in two broad groups, early and late, defined by the time of their appearance during infection (Rabussay and Geiduschek, 1977; Brody et al., 1983; Christensen and Young, 1983). Early proteins, those needed for phage DNA replication, T4 deoxyribonucleotide synthesis, and regulation of phage transcription and translation, are the first gene products to be expressed, and their synthesis continues until about 10 to 12 minutes post-infection. At that time, early gene transcription ceases as phage-modified host RNA polymerase switches to transcription of late genes. Proteins appearing late during phage infection, after about 10 to 12 minutes post-infection, are almost exclusively used for phage virus assembly, packaging of DNA into phage heads, and lysis of the host cell. Transcription of late genes is dependent on at least two events, the onset of phage DNA replication, and modification of the host RNA polymerase by phage-encoded proteins, gene product (gp) 33,

gp45 and gp55. In the absence of either event, T4 late genes are not expressed (Rabussay, 1983; Geiduschek et al., 1983).

Phage DNA replication, described in more detail below, begins about 5 to 7 minutes post-infection and continues until cell lysis, usually 20 to 30 minutes post-infection.

T4 DNA contains the novel deoxyribonucleotide, 5-hydroxymethyldeoxycytidylate (hmdCMP), uniformly distributed throughout phage DNA. As discussed below, cytosine is not modified to hydroxymethylcytosine after replication, rather hmdCTP instead of dCTP is used as a substrate for T4 DNA synthesis. The hydroxymethyl modification prevents clevage of T4 DNA by virtually all but a handful of restriction endonucleases and has made cloning of T4 genes more difficult than ordinary. Moreover, modification of hmdCMP residues in T4 DNA by phage-encoded glucosyltransferases, acts to protect the DNA from certain host restriction systems (Revel, 1983). And, since there are a number of T4-encoded deoxyribonucleases expressed during phage infections that degrade cytosine-containing DNA, hmdCMP provides T4 with a means of identifying self (phage) from non-self (E. coli) DNA (Warner and Snustad, 1983).

Furthermore, a system has evolved in T4 whereby transcription of late genes requires hydroxymethylcytosine-containing templates (Snustad et al., 1983). This template specificity is controlled by the phage alc/unf gene product (acronym for allows late-gene transcription from cytosine-containing DNA and unfoldase, the T4 activity that causes unfolding of the condensed host chromosome), and inactivation of the wild type T4 alc/unf protein is necessary for production of phage with unmodified cytosine-containing DNA (C-DNA).

However, T4 with 100% C-DNA (Fig. 1) can be produced by infections with phage mutant in gene 42 (dCMP hydroxymethyltransferase, gene 56 (dCTPase/dUTPase), alc/unf, denA (endonuclease II), and denB (endonuclease IV) (Kutter and Snyder, 1983).

Meanwhile, by about 5 minutes post-infection, host transcription, translation, and DNA synthesis are shutoff and converted to synthesis of phage products. This cessation of host macromolecular synthesis is thought to be accomplished in part by disruption of cell membrane functions, and by expression of phage products needed to more completely take over host macromolecular synthesis (Snustad et al., 1983).

B. T4 Deoxyribonucleotide Metabolism

Immediately on infection, T4 induces several enzyme activities, which create a new deoxyribonucleotide metabolic pathway tailored to supply dNTPs for phage replication (reviewed by Mathews and Allen, 1983). This host cell-phage hybrid pathway includes three, probably essential, host enzymes, namely, serine hydroxymethyltransferase, dAMP kinase, and (deoxy)ribonucleoside diphosphate kinase, and eight phage-encoded enzymes, namely, ribonucleotide reductase, thioredoxin, thymidylate synthase, thymidine kinase, dCMP deaminase, dCTPase/dUTPase, dCMP hydroxymethyltransferase and dNMP kinase. E. coli and T4 deoxyribonucleotide metabolism are illustrated in Figure 2 and Figure 3, and the reactions catalyzed by the phage-induced enzymes are summarized in Table II.

The addition of these phage proteins to host deoxyribonucleotide

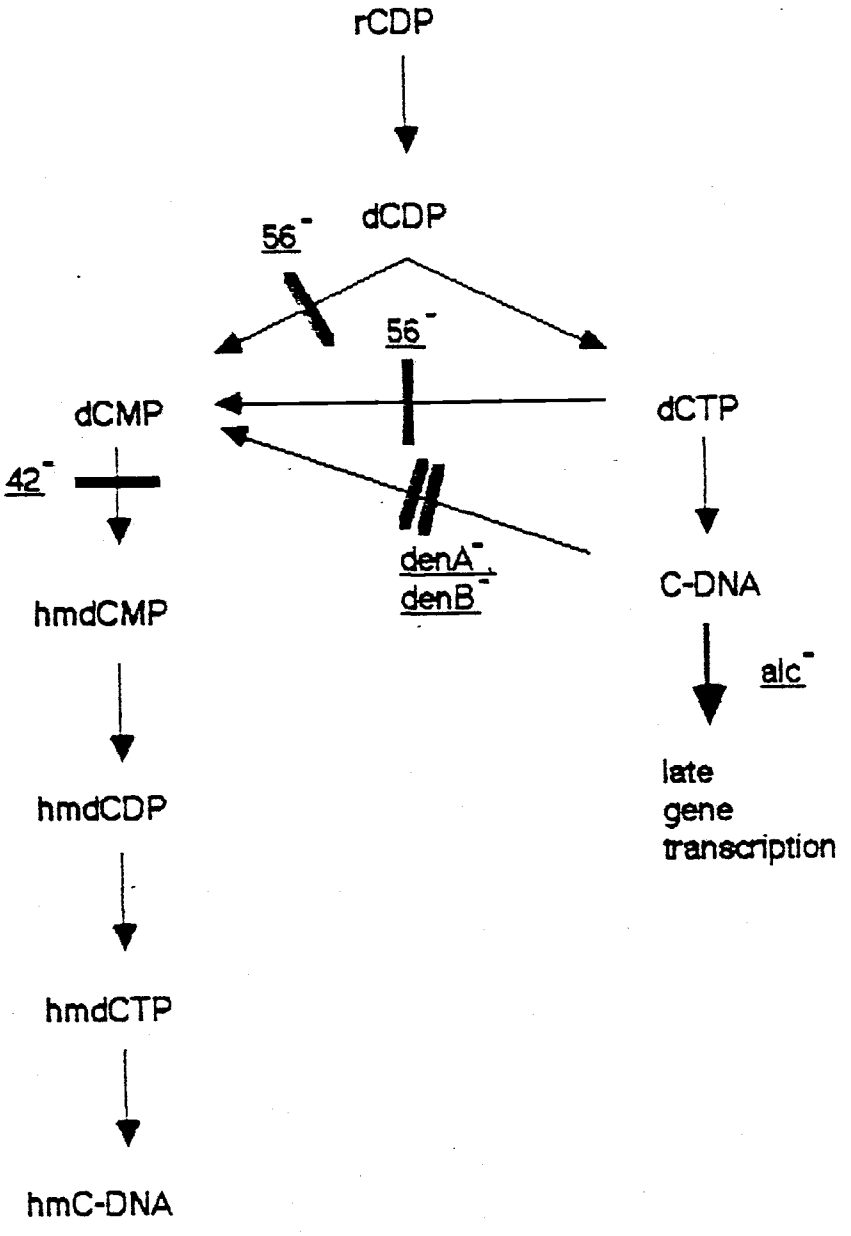


Figure 1. Production of Viable T4 with Cytosine-DNA

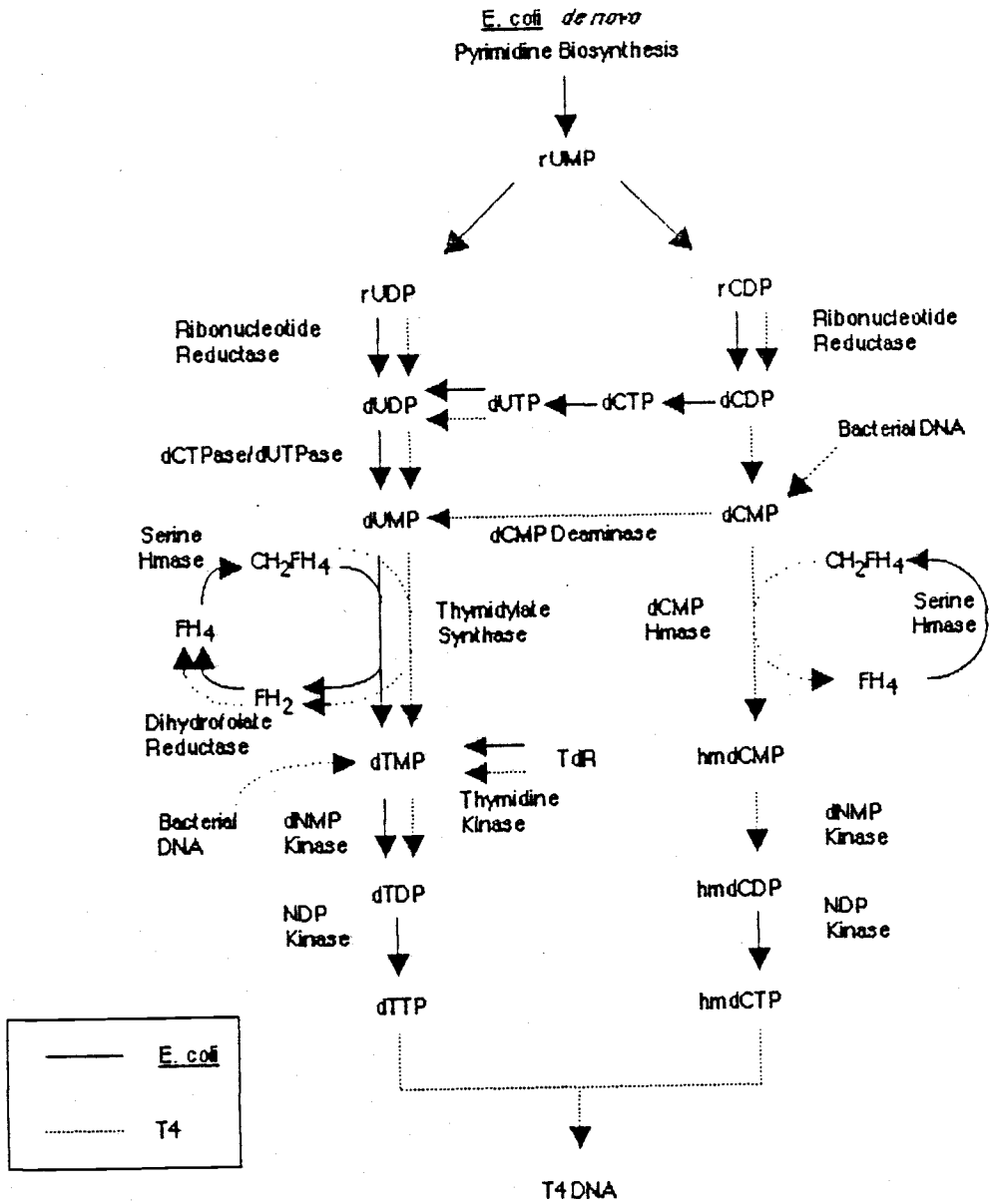


Figure 2. E. coli and T4 Pyrimidine Deoxyribonucleotide Metabolism.

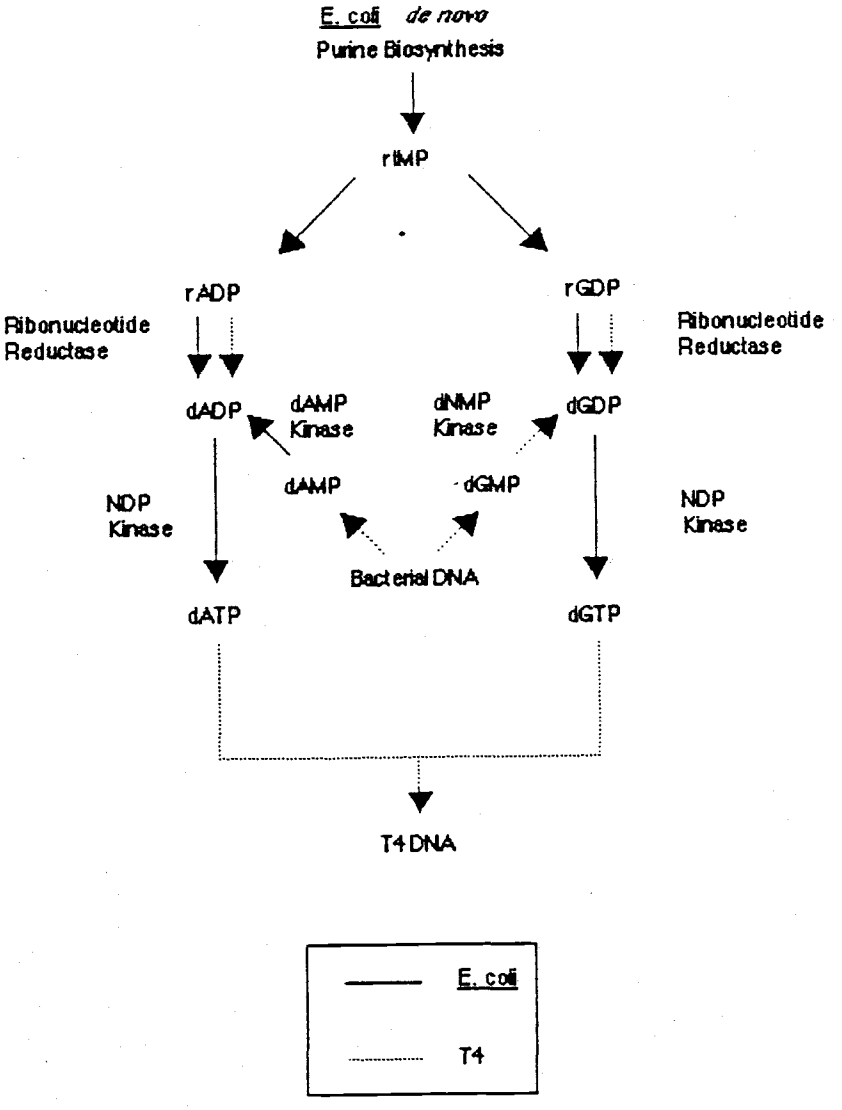
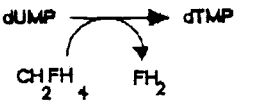
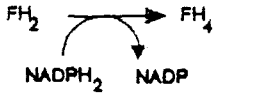
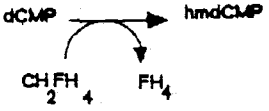
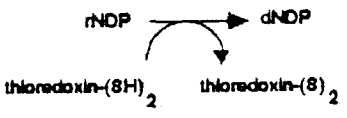
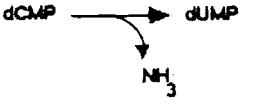
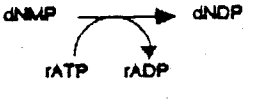
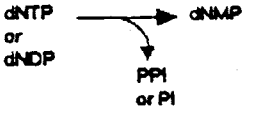


Figure 3. E. coli and T4 Purine Deoxyribonucleotide Metabolism.

Table II. Enzymes of T4 Deoxyribonucleotide Metabolism.

Enzyme	Reaction	Substrates	Km or apparent Km	Turnover [a]
Thymidylate Synthase		dUMP	20 μM [b]	113 [b]
		CH_2FH_4	—	
Dihydrofolate Reductase		FH_2	2.3 μM [c]	4350 [d]
		$NADPH_2$	18 μM	
dCMP Hydroxymethylase		dCMP	600 μM [e]	276 [f]
		CH_2FH_4	100 μM	
Ribonucleotide Reductase		rUDP	100 μM [g]	490 [g]
		rCDP	43 μM	
		rGDP	45 μM	
		rADP	48 μM	
		Thioredoxin		
dCMP Deaminase		dCMP	100 μM [h]	31,605 [h]
				Relative Rate [l]
dNMP Kinase		dUMP	— [l]	0.03
		dTMP	300 μM	1.0
		dGMP	85 μM [l] ≥500	1.0
		hmdCMP	56 μM	0.5
		rATP	0.8 to 5 mM	
dCTPase/dUTPase		dUTP	— [k]	11,143
		dUDP	— [J]	2,229
		dCTP	4.3 μM	8,357
		dCDP	2.4 μM	1,671

a) moles product/moles native enzyme/minute, 37° C, pH 7.0 except for dCMP deaminase and dCTPase/dUTPase which were at pH 8.0 to 8.4
b) Capco, et. al., 1973
c) Erikson, 1971
d) Erikson and Mathews, 1971
e) T6 enzyme, Pizer and Cohen, 1962
f) T4 enzyme, North and Mathews, 1977
g) Berglund, 1972a,b
h) Scocca, et. al., 1969
i) T2 enzyme, Bello and Bessman, 1963
J) T2 enzyme, Zimmerman and Kornberg, 1961
k) T4 enzyme, Price and Warner, 1969

metabolism probably fills three roles. (1) T4 deoxyribonucleotide metabolism must provide for the synthesis of hmdCTP and simultaneously prevent dCTP synthesis. The coordinate action of three T4 enzymes, dCTPase/dUTPase, dCMP hydroxymethyltransferase and dNMP kinase provide this function. (2) For reasons described below, it is unlikely that host deoxyribonucleotide metabolism has the capacity to synthesize enough dNTPs necessary to support T4 DNA replication. Hence, to increase the metabolic capacity, T4 duplicates several host encoded deoxyribonucleotide metabolic enzymes, including ribonucleotide reductase, thymidylate synthase, thymidine kinase, and dihydrofolate reductase. (3) Finally, since T4 DNA is very A:T rich (66%) compared to E. coli (50% A:T), accurate replication of T4 DNA may require synthesis of a different balance of dNTPs as compared to that needed for E. coli DNA replication. This final role of tuning deoxyribonucleotide metabolism to meet the needs of T4 is jointly filled by ribonucleotide reductase and dCMP deaminase. Both of these enzymes are positioned early in deoxyribonucleotide metabolism (Fig. 2) and both are regulated by dNTPs, the end products of deoxyribonucleotide metabolism.

The first step unique to the production of hmdCTP is the synthesis of hmdCMP by the product of T4 gene 42, dCMP hydroxymethyltransferase. The low Vmax and the relatively high Km for dCMP (Table II) make this enzyme, along with thymidylate synthase (Table II), potential bottlenecks in pyrimidine biosynthesis. Both enzymes also use the same single carbon donor substrate for dTMP and hmdCMP biosynthesis, N^5N^{10} methylenetetrahydrofolate, which is synthesized by the host serine hydroxymethyltransferase.

In contrast to thymidylate synthase and dCMP hydroxymethyltransferase, T4 dNMP kinase and host (d)NDP kinase have extemely high reaction rates. For example, purified host (d)NDP kinase is capable of synthesizing about 10^5 moles of (d)NDPs per mole of enzyme per minute (Ingraham and Ginther, 1978). While the host (d)NDP kinase is nonspecific with respect to substrate (it can phosphorylate deoxy- and ribonucleoside diphosphates, including dTDP, dUDP, dADP, dGDP, dCDP, and hmdCDP), the product of T4 gene 1, dNMP kinase, selectively phosphorylates hmdCMP, dTMP, and dGMP, but not dAMP. Furthermore, since dCMP is not a substrate of dNMP kinase, and since the Km for dUMP is about 2 mM, a dUMP concentration not likely to be reached during phage infections, dCDP and dUDP (and ultimately dCTP and dUTP) should not be synthesized via this pathway (Bello and Bessman, 1963). Alternatively, since hmdCMP is not a substrate for host (d)CMP kinase, T4 dNMP kinase and dCMP hydroxymethyltransferase are essential for hmdCTP biosynthesis; inactivation of either protein prevents phage DNA replication, and is lethal for phage growth.

A cell-specific pathway for the synthesis of dUTP and dCTP, however, is left intact during T4 infection. For example, during infections with phage containing the appropriate mutations in deoxyribonucleotide metabolism and T4 deoxyribonucleases, T4 DNA can be synthesized either fully substituted with cytosine for hydroxymethylcytosine (Kutter and Snyder, 1983) or 30% substituted with uracil for thymine (Warner and Duncan, 1978). Protection against significant dCTP or dUTP accumulation during wild type infections, and its subsequent incorporation into phage DNA, is provided by T4 dCTPase/dUTPase (Price and Warner, 1969). Like its cell analog, E.

coli dUTPase (Bertani et al., 1963; Shlomai and Kornberg, 1978), the low Km of T4 dCTPase/dUTPase, less than the Km of other enzymes in deoxyribonucleotide metabolism (Table II), and its relatively high Vmax work to maintain dCTP and dUTP concentrations below the Km of DNA polymerase. DNA replication can be detected during infections with T4 gene 56 mutants (Kutter and Wiberg, 1968; Warner and Hobbs, 1967; Wiberg, 1967), but phage DNA does not accumulate and is rapidly degraded by nucleases specific for cytosine containing DNA.

A second function of dCTPase/dUTPase is to provide dUMP and dCMP for utilization by dCMP hydroxymethyltransferase, dCMP deaminase and thymidylate synthase (Price and Warner, 1969). Unlike E. coli dUTPase (Bertani et al., 1963), dCTPase/dUTPase also hydrolyzes pyrimidine deoxyribonucleoside diphosphates and should hydrolyze the products of ribonucleotide reductase, dUDP and dCDP (Table II). In contrast, pyrimidine rNDPs in uninfected host deoxyribonucleotide metabolism are probably phosphorylated to dNTPs before E. coli dCTP deaminase and dUTPase turnover dCTP to dUMP.

Phage production is depressed but not prevented during infections by T4 mutant in ribonucleotide reductase, thymidylate synthase, dCMP deaminase and dihydrofolate reductase. Hence, these T4 gene products are usually thought of as "non-essential" in comparison to the essential genes dCTPase/dUTPase, dCMP hydroxymethyltransferase and dNMP kinase.

The idea that these non-essential enzymes function to increase dNTP metabolic activity, comes from several observations. (1) Typically, there is at least a 5- to 10-fold increase in each of these deoxyribonucleotide metabolic enzyme activities during T4 infections,

as compared to existing host cell activities (dihydrofolate reductase: Hall, 1967; Mathews, 1967; thymidylate synthase: Flaks and Cohen, 1959; ribonucleotide reductase: Yeh et al., 1969). The sole exception is T4 dCMP deaminase, whose closest metabolically equivalent enzyme in E. coli deoxyribonucleotide metabolism is dCTP deaminase. (2) Phage production during infections of T4 mutant in these enzyme activities is about 50% of wild type infections (ribonucleotide reductase: Yeh and Tessman, 1972; thymidylate synthase: Mathews, 1965; dCMP deaminase: Hall and Tessman, 1966; dihydrofolate reductase: Hall, 1967; Mathews, 1967). Conceivably, decreased phage production could be attributed to losing an unexpected function outside of dNTP metabolism, but filled by one of the deoxyribonucleotide metabolic enzymes. For example, T4 thymidylate synthase and dihydrofolate reductase both are structural components of the phage virion baseplate, and mutations in these two enzymes are known to affect the stability of phage virions (see Kozloff, 1983). But in the case of infection by thymidylate synthase and dihydrofolate reductase mutants, phage DNA synthesis is also reduced about 50% relative to wild type infections (Mathews, 1967; Mathews, 1965). Since the rate of phage DNA synthesis is about 5 to 10 times greater than that of E. coli, and by the time of cell lysis at least 10 host cell-DNA equivalents of T4 DNA are synthesized, it should not be surprising that host enzyme activities might not be capable of pacing the demand for dNTPs during phage infections.

Ribonucleotide reductase and dCMP deaminase are strategically positioned to control the relative concentrations of deoxyribonucleotides synthesized during deoxyribonucleotide

metabolism. Ribonucleotide reductase mediates the commiting step to de novo deoxyribonucleotide biosynthesis by controlling reduction of ribonucleoside diphosphates to deoxyribonucleoside diphosphates; and is found in virtually all eucaryotic and procaryotic cells investigated (Thelander and Reichard, 1979; Nutter and Cheng, 1984) as well as three viral systems: vaccinia (Slabaugh and Mathews, 1984; Slabaugh et al., 1984) and herpes viruses (Cohen, 1972), and bacteriophage T4 (Yeh et al., 1969; Berglund et al., 1969). Moreover, all ribonucleotide reductases studied to date characteristically share elaborate allosteric regulation.

In comparing cellular with the virus-specified forms of ribonucleotide reductase (Fig. 4), the more complex system of allosteric regulation is seen in those purified from mammalian cells and E. coli (Thelander and Reichard, 1979; Nutter and Cheng, 1984). These enzymes appear to have two classes of regulatory sites: activity sites, which act generally as an on/off switch for enzyme activity where rATP is the positive effector and dATP is the negative effector; and specificity sites, where dTTP, dATP, and dGTP serve to modulate the preference for its substrates rGDP, rADP, rCDP and rUDP. Ribonucleotide reductases from herpes virus infections or T4 infections, however, differ from the enzymes described above, in that these virus-specified enzymes seem to lack the activity site, which determines the overall ribonucleotide reductase activity (Averett et al., 1983; Berglund, 1972b). In essence, herpes and T4 ribonucleotide reductases are always turned "on" for ribonucleotide reduction and cannot be turned "off". Furthermore, in the case of the T4 enzyme, the deoxyribonucleotide effectors elicit a different enzyme preference

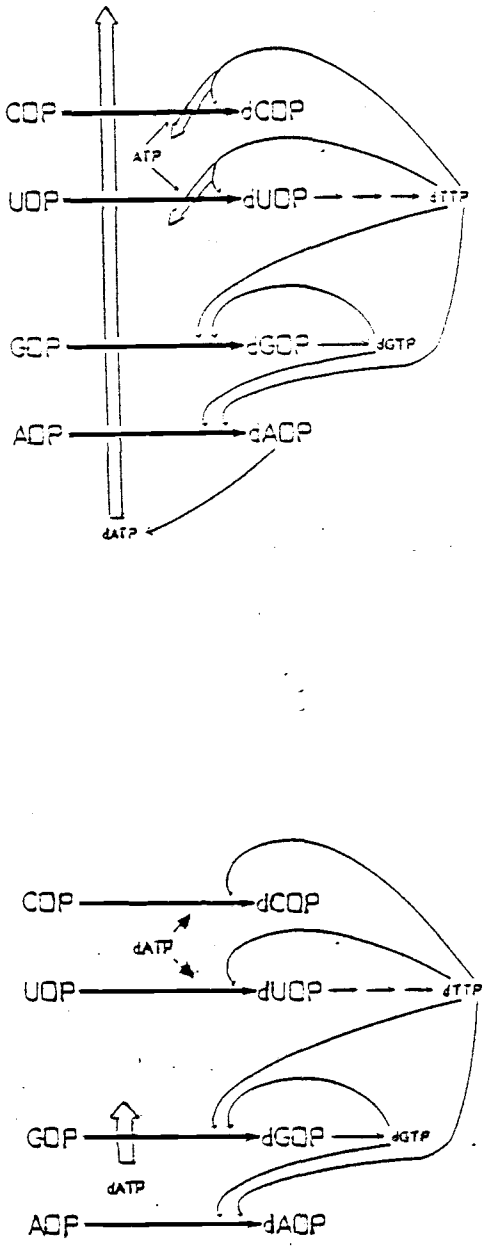


Figure 4. Regulation of E. coli and T4 Ribonucleotide Reductases (adapted from Larsson and Reichard, 1966). The unshaded arrows, associated with dATP and dTTP, represent inhibitory effects. Solid arrows associated with dTTP, dGTP, dATP, and ATP represent stimulation of enzyme activity towards that substrate.

for substrates than the E. coli enzyme (Larsson and Reichard, 1966a,b; Berglund, 1972b; Berglund, 1975).

In mammalian cells, and T4 infections, the deamination of dCMP to dUMP by dCMP deaminase is the major pathway providing substrate for deoxythymidylate biosynthesis (Jackson, 1978; Chiu et al., 1977). As mentioned above, this pathway in E. coli and Salmonella tyhimurium is via dCTP deaminase to dUTP, which is hydrolyzed to dUMP by the cell dUTPase (Neuhard, 1968; Karlström and Larsson, 1967). All dCMP and dCTP deaminases characterized to date share similar features of enzyme regulation, and are activated by dCTP (or hmdCTP) and inhibited by dTTP. Consequently, regulation of these branch points in deoxyribonucleotide metabolism can determine the ratio of dCTP (and hmdCTP) to dTTP synthesized in these organisms.

C. T4 DNA Replication

There are over 200 phage equivalents of T4 DNA synthesized during its 20- to 30-minute life cycle, which is almost ten times the amount of DNA synthesized by E. coli in a comparable amount of time. The high rate of DNA replication in T4 infections is achieved in most part by having more replication forks than E. coli (Werner, 1968). To sustain these high replication rates, T4 encodes a set of proteins suited specifically for phage DNA synthesis. Some 20 different T4 gene products have been identified by the effects of mutations in those genes on phage DNA replication, and grouped into four functional categories. These groups are: DO (DNA-negative or no DNA synthesis) genes 1 (dNMP kinase), 42 (dCMP hydroxymethyltransferase), 43 (DNA

polymerase), 62, 44, and 45 (DNA polymerase accessory proteins); DS (some DNA synthesis) genes 56 (dCTPase/dUTPase), 30 (DNA ligase), 32 (helix destabilizing protein), and 41 (DNA helicase); DD (delayed DNA synthesis) genes 39, 52, 60 (T4 topoisomerase subunits), 61 (RNA primase), and 63 (RNA ligase); and DA (arrested DNA synthesis) genes 46, 47, and 59 (Warner and Hobbs, 1967).

There are at least two mechanisms by which T4 DNA replication initiates during phage infections (Mosig, 1983b; Kozinsky, 1983). At low multiplicities of infection, i.e. one phage per cell or less, fairly discrete origins of replication have been identified on the T4 chromosome (Kreuzer and Alberts, 1985; Kreuzer and Alberts, 1986; Mosig, 1983b; Kozinsky, 1983), which depend on E. coli RNA polymerase for initiation of replication. As replication progresses and daughter duplex DNA molecules accumulate in the cell, a second mechanism, involving recombination, comes to predominate (Mosig, 1983b). At high multiplicities of infection, and late during T4 infection, when many copies of the T4 genome are present, replication initiation is believed to proceed by both schemes.

Since T4 DNA is a linear molecule, replication of an entire chromosome will leave two single-stranded 3' ends. While leading strand replication should essentially run-off the ends of the template DNA strands, lagging strand synthesis should leave part of the 3' ends unreplicated. Initiation by recombination is thought to take advantage of these single-stranded ends, by using them to initiate replication on other T4 chromosomes, and complete the replication of the ends.

Of the 20 gene products, mentioned above, involved in phage DNA

replication, a minimum of 5 phage-encoded proteins (DNA polymerase, single stranded binding protein gp32, and the DNA polymerase accessory proteins gp45 and gp44/62) are required to support replication of single-stranded DNa templates in vitro (reviewed by Nossal and Alberts, 1983). Replication of double-stranded templates requires the presence of at least two additional proteins, a DNA helicase, either gp41 or gpdda, and the T4 RNA primase, gp61. During cell-free replication supported by balanced dNTP concentrations, the seven-protein complex (schematically represented in Figure 5) replicates small, natural DNA templates at about the same rate as during phage infections, and with comparable spontaneous mutation rates (Nossal and Alberts, 1983; Sinha and Goodman, 1983).

Most of the gene products in the 5-protein replication complex serve to increase the processivity (the number of nucleotides synthesized by DNA polymerase before dissociating from the template) and the rate of T4 DNA polymerase movement on the template. T4 DNA polymerase is a monomeric enzyme containing both a 5'-to-3' DNA-dependent polymerase and a 3'-to-5' DNA exonuclease activity. It is capable of synthesizing short, DNA fragments on single-stranded templates, and on its own cannot catalyze strand-displacement synthesis on double-stranded templates.

As mentioned above, in addition to T4 DNA polymerase, a core group of 4 proteins is required to facilitate DNA synthesis on single-stranded templates. T4gp32, the T4 single-stranded binding protein, binds cooperatively to single stranded nucleic acids and functions during T4 replication to stabilize and maintain single-stranded DNA in front of the replication fork. Phage DNA

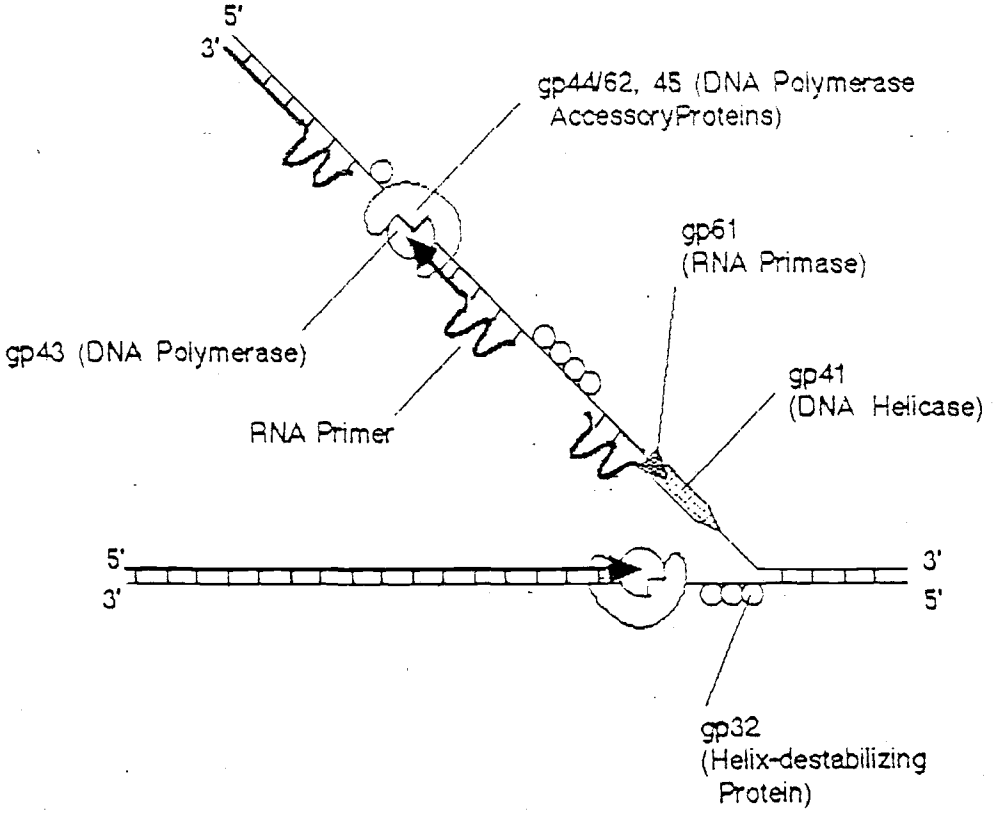


Figure 5. Organization of the T4 Replication Fork (from Nossal and Alberts, 1983).

polymerase accessory proteins gp44, gp62, and gp45 form a tight multi-protein complex stabilizing DNA polymerase on the template, and are thought to form a sliding clamp, holding polymerase on the template, and thereby increasing processive DNA synthesis. The stability of the Polymerase/44/62/45 complex is dependent on regular rATP hydrolysis. Apart from its function in maintaining single-stranded template structure, movement of the replication complex is also stimulated by gp32. Finally, the product of gene 61, RNA primase, is responsible for the synthesis of RNA primers for lagging strand synthesis, and is strongly enhanced in the presence of gp41.

Either of two single-stranded dependent helicases are required for rapid in vitro replication of double-stranded templates. One of these, gp41, has maximum activity in the presence of the DNA polymerase accessory proteins and gp61, and can unwind about 50 to 400 basepairs of duplex DNA with the expenditure of rGTP or rATP. Another helicase, the product of the dda gene, unwinds longer regions of DNa with the expenditure of rATP and can substitute for the helicase activity of gp41 during in vitro replication. Gene product dda differs from gp41 in that gpdda acts stoichiometrically, with one protein molecule needed for every two basepairs unwound. A more practical distinction between these two helicases can be made on the observation, that, regardless of gp41 presence, replication forks in vitro cannot proceed past template bound RNA polymerase when gpdda is absent. Apparently, gpdda acts as a cowcatcher for the T4 replication complex and can displace certain template-bound obstacles.

D. T4 DNA Repair

Since T4 quickly inactivates host macromolecular synthesis, during infections, phage DNA repair (reviewed by Bernstein and Wallace, 1983) should be independent of inducible host repair systems. One example that is consistent with this prediction is seen in the different spectrum of mutations observed for T4 and E. coli induced during thymidylate deprivation. Whereas thymidylate deprivation in uninfected E. coli, which is dependent on an inducible recA error-prone repair system (Bridges et al., 1968) and results in several classes of mutations (Holmes and Eisenstark, 1968; Pauling, 1968; Bresler et al., 1973; Kunz and Glickman, 1985), only AT-to-GC transitions are detected by thymidylate deprivation during T4 infections (Drake and Greening, 1970; Bernstein et al., 1972; Smith et al., 1973). However, T4 does utilize at least two host-specific repair systems (Bernstein and Wallace, 1983). In addition to phage endonuclease V-mediated excision-repair, thymidine dimers caused by ultraviolet radiation can be repaired by the host photoreactivation system. Uracil in E. coli DNA, occurring either by deamination of template cytosines or by incorporation of dUMP, is removed by E. coli uracil-N-glycosylase. Mutations in the E. coli ung gene, inactivating uracil-N-glycosylase, are required in order to synthesize phage or E. coli DNA in which thymidine has been replaced with uracil (Warner and Duncan, 1978).

Currently, there are three phage-specific repair systems identified: UV-excision repair, multiplicity reactivation, and post-replication-recombinational-repair (Bernstein and Wallace, 1983).

Excision repair of thymidine dimers in T4 DNA is mediated by the gene product of the phage denV gene, endonuclease V. This bifunctional protein contains both a glycosylase activity, responsible for cleaving the N-glycosyl bond between deoxyribose and thymidine on the 5' side of thymidine dimers, and an apyrimidinic/apurinic endonuclease which cleaves the phosphate bond between the apyrimidinic/apurinic site and the next (3') nucleotide. The thymidine dimers are then thought to be removed by an unidentified exonuclease or possibly host DNA polymerase I, and a DNA patch is resynthesized by host DNA polymerase I and ligated by T4 DNA ligase.

Two phage-specific recombinational repair pathways, Post-Replication-Recombinational-Repair (PRRR) and Multiplicity-Reactivation (MR), are active during T4 infections. MR is distinguished by recombination between infecting genomes, prior to replication, to correct defective parental chromosomes. This is typically observed during infections of more than one phage per cell and depends on several enzyme activities common to PRRR. There are, however, several activities unique to the MR pathway, including T4 endonuclease V, T4 topoisomerase, and E. coli DNA polymerase I and recA protein. One final distinguishing trait unique to MR, is that recombination is hyperstimulated in comparison to normal recombinational activity and PRRR.

PRRR, on the other hand, is detected during infections of one phage per cell or less, and hence depends on phage DNA replication to produce daughter chromosomes for recombinational correction of mutations. PRRR requires uvsX protein activity as well as T4 DNA ligase, the products of genes 1, 42, and 56, and T4 DNA polymerase,

gp32 and many of the polymerase accessory proteins.

Repair of base mismatches in T4 DNA has been detected, and is believed to rely in part on phage gene products (Berger and Pardoll, 1976). This repair system, however, is uncharacterized and its contribution to T4 mutagenesis has not been described.

As described above, T4 does not appear to have an inducible error-prone repair system similar to that of E. coli. There is evidence, however, that error-prone repair by T4 gene products is responsible for some phage mutations. For example, the mutants most often recovered after UV irradiation are GC-to-AT transitions (Meistrich and Drake, 1972). If, as is believed to be the case, thymidine dimers are the dominant lesion introduced by UV treatment, then the GC-to-AT transitions must be occurring at sites near, but not opposite the dimers, and are a result of error-prone repair of these lesions. Furthermore, T4 DNA polymerase has been implicated in this repair pathway, since some antimutator polymerases inhibit UV mutagenesis (Yarosh et al., 1980). At least three other T4 gene products are known to participate in error-prone repair pathways, namely, the gene products of uvsW, uvsY, and uvsX, as defined by the observation that mutations in these genes also reduce UV mutagenesis in T4. Finally, the T4 hm (hypermutable) mutant stimulates spontaneous mutation rates, causes increased UV mutagenesis, and has heightened sensitivity to thymineless and base analog mutagenesis (Green and Drake, 1974; Drake, 1973; Smith et al., 1973). The hm allele has been mapped to the gene-43 carboxy terminus (J. Drake, personal communication), but the DNA polymerase activities altered by this mutation have not been identified.

E. Determinants of Replication Fidelity

1. Deoxyribonucleotides

Evidence from cell-free replication systems and intact cell systems has established that dNTP pool imbalances can promote misincorporation of deoxyribonucleotides into replicating DNA (Kunz, 1982; de Serres, 1985). For most examples studied the mechanism leading to base misincorporations during dNTP imbalances is through competition between correct and incorrect nucleotides for insertion into the growing DNA strand. Hydrogen-bonded mispairs leading to transition mutations (AT-to-GC or GC-to-AT) are believed to be accommodated in the DNA helix if one of the participating nucleotides in the mispair assumes a rare tautomeric or ionized form (Watson and Crick, 1953a,b). However, to accommodate hydrogen-bonded mispairs leading to transversions (AT-to-TA, GC-to-CG, AT-to-CG, and GC-to-TA) without drastic distortions of the helix or forming unlikely hydrogen bonds, Topal and Fresco (1976) suggested that the most favorable conformation of incorporated nucleotides are isomers, of tautomeric or ionized forms of the bases, with the base rotated about the N-glycosidic bond into the syn position. Moreover the increased ability to stimulate transitions by two base analogs, bromodeoxyuridine and 2-aminopurine, is believed to reflect their higher equilibrium favoring ionized and tautomeric form of the bases (Freese, 1959).

Cell-free replication systems, comprised of the purified components required for *in vitro* DNA replication, namely, DNA

polymerase, accessory proteins, a defined natural template, and the four dNTPs, have made it possible to carefully study the relationship between dNTP imbalances and base misincorporations in vitro (Weymouth and Loeb, 1978; Fersht, 1979; Hibner and Alberts, 1980). These cell-free systems mimic in vivo replication in several ways, for example, similar replication rates, spontaneous mutation frequencies, and template-specific effects for base substitutions. In studies using the T4 seven-protein replication system, and measuring reversion at various amber mutations in the small bacteriophage ϕx174 template, several researchers have described a linear relationship between increasing G/A, C/T, A/T, and G/T deoxyribonucleotide pool imbalances, and the frequency of mutations brought about through base misincorporation (Hibner and Alberts, 1980; Sinha and Haimes, 1981; Sinha and Goodman, 1983). These imbalances, however, are not equal in their ability to cause misincorporation. By measuring mutagenesis when systematically varying the dNTP pool imbalances during in vitro replication, the relative order of mispaired nucleotides in the DNA has been determined to be G:T>(A:C≈A:G≈A:A)>(C:T≈T:T) (Sinha and Goodman, 1983). Thus, AT-to-GC transitions in vitro can be expected to occur most frequently by G:T mispairs, which in turn are promoted by an increasing G/A dNTP pool bias. In comparable experiments, misincorporations leading to transversions occurred much less frequently than transition mutations, and AT-to-TA and AT-to-CG transversions were found to involve purine:purine rather than pyrimidine:pyrimidine mismatches (Sinha and Haimes, 1981; Sinha and Goodman, 1983).

For the most part, deoxyribonucleotide pools in intact cells can

be manipulated three ways: supplementing intracellular nucleotide pools by the addition of exogenous nucleosides, inhibiting nucleotide synthesis with an antimetabolite directed against steps in nucleotide metabolism, and altering nucleotide pools as a consequence of mutations in nucleotide metabolism. Of the pathways synthesizing dNTPs, pyrimidine deoxyribonucleotide synthesis, especially thymidylate metabolism, has been the most frequent target to induce imbalanced dNTP pools in both procaryotes and eucaryotes (Kunz, 1982).

Thymidylate starvation in E. coli results in expanded dCTP and dATP pools, unchanged dGTP pools, and reduced dTTP pools (Neuhard and Munch-Petersen, 1966). While, in E. coli, AT-to-GC mutations are the predominant mutation induced as a result of these imbalanced dNTP pools, GC-to-AT transitions, transversions, and frameshift and deletion mutations are also recovered (Holmes and Eisenstark, 1968; Pauling, 1968; Bresler et al., 1973; Kunz and Glickman, 1985). In comparison, AT-to-GC transitions represent the only mutation induced during thymineless mutagenesis of replicating T4 (Drake and Greening, 1970; Bernstein et al., 1972; Smith et al., 1973). In both cases, simple misincorporation of dCMP for dTMP into replicating DNA, promoted by the dCTP/dTTP imbalance, can explain the increased AT-to-GC transitions. The difference in the kinds of mutations seen in T4 and E. coli during thymidylate starvation are likely caused by an error-prone repair system induced in E. coli, but not T4. Mutagenesis induced by thymidylate starvation in E. coli is known to be dependent on the E. coli recA-dependent error-prone repair system, and mutations that interfere with its induction, e.g. lexA mutants, prevent thymineless mutagenesis (Bridges et al., 1968).

Likewise, growth in high concentrations of thymidine, causing superabundant dTTP pools, is mutagenic for mammalian cells (Bradley and Sharkey, 1978), E. coli (Kunz, 1982), and T4 (Bernstein et al., 1972; DeVries and Wallace, 1982). In T4, however, excess thymidine stimulates not only GC-to-AT transitions, but also AT-to-GC and frameshift mutations.

Since T4 normally requires hmdCTP, instead of dCTP, as a substrate for phage DNA synthesis, mutations in T4 gene 42, dCMP hydroxymethyltransferase, can be used to inhibit hmdCMP synthesis, and subsequently reduce hmdCTP pools. In fact, GC-to-AT transitions are increased during infections by temperature-sensitive (ts) gene 42 mutants at temperatures thought to partially inhibit dCMP hydroxymethyltransferase activity (Chiu and Greenberg, 1983; Williams and Drake, 1977). This result is consistent with the idea that increased substrate competition between intracellular dTTP and reduced hmdCTP pools is causing misincorporation of dTMP into T4 DNA. However, some transversions and frameshift mutations, not easily explained by substrate competition, are also observed during ts42 infections. As a consequence, Williams and Drake (1977) suggested that these latter effects are a result of altered interactions between the T4 DNA replication complex and the T4 dNTP synthesizing complex. In principle, the altered protein conformations transmitted between the complexes would result in loss of replication fidelity by changes in replication protein activities. Since then, genetic evidence has been presented for in vivo protein-protein interactions between dCMP hydroxymethyltransferase and DNA polymerase (Chao et al., 1977), and dihydrofolate reductase and gp41, and gp61 (Macdonald and Hall, 1983).

Mutations in (deoxy)ribonucleotide metabolism of mammalian cells, causing deranged dNTP pool imbalances, also result in the acquisition of mutator phenotypes. In mammalian cells, as well as T4, a large fraction of the dUMP used for dTMP biosynthesis is synthesized by deamination of dCMP by dCMP deaminase. Loss of cellular dCMP deaminase causes increased dCTP accumulation in the cell and decreased dTTP pools (Weinberg et al., 1981; de Saint Vincent et al., 1980). A comparable dCTP/dTTP pool imbalance is generated by mutations in rCTP synthetase that decrease the enzyme's sensitivity to feedback inhibition by dCTP (Meuth et al., 1979; Trudel et al., 1984; Aronow et al., 1984). In both instances, a strong mutator phenotype is associated with the deranged dNTP pools. Likewise, mutations in cellular ribonucleotide reductase leading to dNTP pool imbalances have proven to be mutagenic (Ullman et al., 1980; Weinberg et al., 1981; Roguska and Gudas, 1984). However, the structures of mutant alleles in these mammalian cell lines are not described, and thus, it is difficult to limit explanation of the increased mutation rates exclusively to base misincorporation from substrate competition, and not induction of error-prone repair systems.

In Salmonella typhimurium and E. coli, dCTP deaminase provides the same funnel for the biosynthesis of dUMP from deoxycytidine nucleotides (Karlström and Larsson, 1967; Neuhard, 1968). Mutations in cdd, the structural gene for dCTP deaminase, induce deoxyribonucleotide pool imbalances comparable to those measured in mammalian cells mutant in dCMP deaminase or rCTP synthetase (O'Donovan et al., 1971). However, it has not been reported whether cdd mutants also confer a mutator phenotype.

Mutant alleles of nucleotide metabolism genes that confer antimutator phenotypes, resulting in reduced mutation rates, are infrequently described. The only example of an antimutator effect in T4 deoxyribonucleotide metabolism is with gene 42 mutants. During ts42 infections where GC-to-AT mutations were increased, AT-to-GC mutation rates were simultaneously reduced (Williams and Drake, 1977). In this case, it is believed the AT-to-GC antimutator effect reflects the occurrence of fewer A:C mispairs, brought about through the reduced hmdCTP pool. Geiger and Speyer (1977) have isolated an E. coli conditional antimutator that maps into the purB gene, the structural gene encoding the enzyme adenylosuccinyl lyase. This enzyme of de novo purine ribonucleotide metabolism catalyzes the removal of succinate from 5-aminoimidazole-4-N-succinocarboxamide-ribotide, and also catalyzes the synthesis of adenylate by removal of fumarate from adenylosuccinic acid. At temperatures above 30°C, presumably inactivating adenylosuccinyl lyase, these cells have reduced dATP, dGTP, and dTTP pools, enlarged dCTP pools, and are auxotrophic for adenine (Lyons et al., 1985). But the mechanism by which this dNTP pool imbalance reduces mutagenesis has not been described. Recently, Quoñones and Piechocki (1985) have isolated several other antimutator E. coli strains, some of which are auxotrophic for purine or serine, and may be mutant in nucleotide metabolic genes.

2. Replication Proteins

Mutant alleles of several T4-encoded replication proteins are

known to confer mutator and antimutator phenotypes. The best studied example is that of T4 DNA polymerase (gene 43). Speyer (1965) first identified temperature-sensitive T4 gene 43 mutants that had increased mutation rates for base misincorporations. Since then, several different gene 43 mutants have been described that confer either mutator or antimutator phenotypes for both base substitutions and frameshift mutagenesis (Speyer et al., 1966; Drake and Allen, 1968; de Vries et al., 1972; Reha-Krantz and Bessman, 1981; Ripley and Shoemaker, 1983; Ripley et al., 1983). These observations have been extended to include DNA polymerases from E. coli (Caukell and Yanofsky, 1970; Hall and Brammer, 1973; Vaccaro and Siegel, 1975; Siegel and Vaccaro, 1978), and herpes virus (Hall et al., 1984).

Purification and characterization of these mutant polymerases demonstrated that the mutator or antimutator properties were associated most frequently with altered DNA polymerase 3'-to-5' exonuclease activity (Muzyczka et al., 1972; Hershfield, 1973; Lo and Bessman, 1976a; Reha-Krantz and Bessman, 1977), and less frequently with an impaired ability to insert correct nucleotides into growing DNA strands (Lo and Bessman, 1976b) or impaired ability for strand displacement synthesis (Gillin and Nossal, 1976). Further studies with these mutant polymerases led to the discovery that the important parameter in determining whether a polymerase will exhibit a mutator or antimutator phenotype is the nuclease/polymerase ratio of the enzyme (Bessman et al., 1974; Goodman et al., 1974; Galas and Branscomb, 1978; Clayton et al., 1970. This ratio is obviously increased in antimutator polymerases, relative to the wild type polymerase, with increased 3'-to-5' exonuclease activities, and the

ratio is decreased in mutator polymerases that have attenuated exonuclease activities. Likewise, conditions inhibiting the progress of polymerase down the template, either by mutations in the enzyme or during limiting substrate concentrations, will effectively increase the nuclease/polymerase ratio and cause increased proofreading.

Mutant alleles of phage DNA replication genes 32 (DNA unwinding protein), 41 (DNA helicase), and 44, 62 and 45 (DNA polymerase accessory proteins) have also been described that stimulate base substitutions (Bernstein, 1971; Koch et al., 1976; Watanabe and Goodman, 1978; Mufti, 1979), as well as alleles of genes 32 and 44 which stimulate frameshift mutagenesis (Bernstein, 1971). Moreover, when tested on the su^+1 amber suppressing bacterium E. coli CR63, the gene 32 amber mutant amE315 and the gene 41 amber mutant amNG18 have been shown to be weakly antimutagenic for AT-to-GC transitions (Watanabe and Goodman, 1978). Exactly how these mutant proteins influence replication fidelity is not known. But, considering the intimate contact between these proteins at the replication fork, the most straightforward hypothesis is that altered interactions between the mutant protein and DNA polymerase affects the polymerase 3'-to-5' exonuclease activity or decreases its ability to discriminate between correct and incorrect deoxyribonucleotide substrates.

3. Template Effects

Perhaps the least understood factor in replication fidelity has been the function of template sequences in determining local mutagenesis. Template sequences certainly influence not only the type

of mutagenic event occurring, base substitutions versus deletions and frameshifts, but also the frequency with which mutations occur. Site specific variability, with respect to mutagenesis, has been observed in many organisms, but perhaps the best example studied so far was the recovery and mapping of spontaneous and induced mutants in the T4 rII genes (Benzer, 1961). In that study, mutations were found to be nonrandomly distributed throughout the rII genes, with mutations occurring more frequently at some sites (mutational hotspots) than at others. The sequence of one such hotspot, located at the beginning of the rIIB gene, has been determined to be -TGGCAA-, where the hypermutable bases are the central -GC- dinucleotide (Singer, 1984).

Other template effects, namely, the influence of neighboring bases in nonsense codons, on base sustitution mutagenesis, have been described by Koch (1971), and others (Salts and Ronen, 1971; Ronen and Rahat, 1976; Ronen et al., 1976; Ronen et al., 1978; Ronen and Halevy, 1980). And, in those studies it was determined that base substitutions occur more frequently when the adjacent 5' base is a cytosine rather than a thymine. Finally, evidence has recently been presented that base substitution mutagenesis can be influenced not only by adjacent nucleotides, but also by sequences a dozen nucleotides removed (Conkling et al., 1980; Sugino and Drake, 1984).

In contrast to base substitution mutagenesis, the mechanisms for frameshift and deletion formation in T4 DNA are much better understood. Streisinger et al. (1966) observed that certain frameshift mutations occurred frequently in runs of poly-dA about 5 bases long. This observation has since been confirmed by Owen et al. (1983), and extended to include deletions formed in regions of

sequence duplications and direct repeats. Streisinger et al. (1966) went on to propose that misalignment of template sequences in the poly-dA runs, could result in either addition or loss of one or more nucleotides. The template misalignments could occur through recombinational intermediates or mispairing at the ends of DNA molecules.

Recently, quasi-palindromic DNA sequences in T4 DNA were shown to have a role in production of frameshift and base substitution mutations (de Boer and Ripley, 1984). Ripley (1982) predicted from existing sequence data, that replication through imperfect stem-loop structures in DNA could result in deletion or addition of nucleotides. As shown in Figure 6a, depending on the strand utilized in the imperfect palindrome, either deletions (upper pathway) or additions (lower pathway) can be formed. Alternatively, replication can undergo strand-switching, where the displaced DNA strand complementary to the template, transiently becomes the replicative template (Fig. 6b). Resolution or repair of these branched molecules, involving the formation of the imperfect palindrome, will then lead to an insertion or deletion of DNA sequences.

F. Present Work

To fully understand mutagenesis induced by dNTP pool imbalances *in vivo*, one must know (1) structures of mutant sites at the nucleotide sequence level, and (2) quantitative relationships between dNTP pool changes and mutation frequencies. Such relationships are probably not clear-cut, as they are in purified *in vitro* systems, since factors

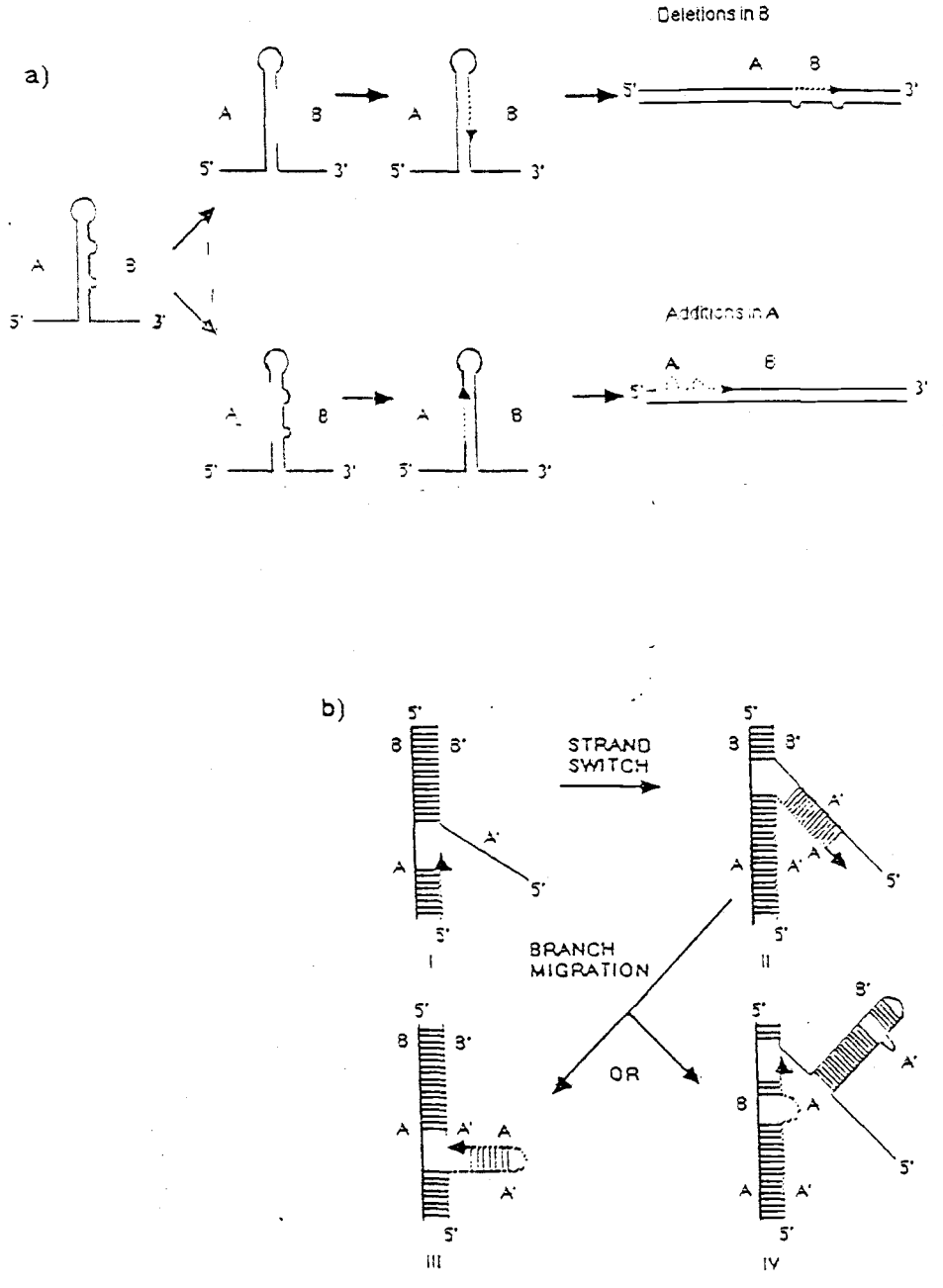


Figure 6. DNA Structures Leading to Deletion and Insertion Mutagenesis (from Ripley, 1982).

other than dNTP competition at insertion sites almost certainly contribute to mutagenesis *in vivo*. Moreover, effective dNTP concentrations at replication sites probably differ from those estimated from dNTP pool measurements (Mathews and Sinha, 1982). The work described herein relied on three assays to probe these relationships between *in vivo* mutagenesis and dNTP pools.

Intracellular dNTP pools were measured by an enzymatic assay using purified *E. coli* DNA polymerase I, defined alternating copolymer templates, and labelled dNTP complementary to the dNTP being measured. Figure 7 illustrates an assay for dTTP, present in a cell sample containing all four non-radioactive dNTPs, and added [^{3}H]dATP. After the polymerization reaction is completed, limited by the amount of dTTP in the cell extracts, the acid precipitable counts are compared to standard curves generated from similar reactions, but using known concentrations of dTTP. This assay is faster and more sensitive (capable of measuring about 0.5 pmol to 100 pmol of dNTP per reaction) then measuring dNTP concentrations by HPLC, or measuring dNTPs extracted from cells whose nucleotide pools are labelled to equilibrium with [^{32}P]-orthophosphate.

The *rII* genes have been described by one phage geneticist as "3600 characters in search of a function" (Drake, 1983). While these genes and their mutant alleles have seen extensive use by molecular biologists, little more is known today about their function during phage infections than was known 40 years ago, when they were discovered by Hershey (1946). In spite of this, there are several characteristics that make the *rII* genes close to ideal for studying mutagenesis. First, there are many well-characterized *rII* alleles

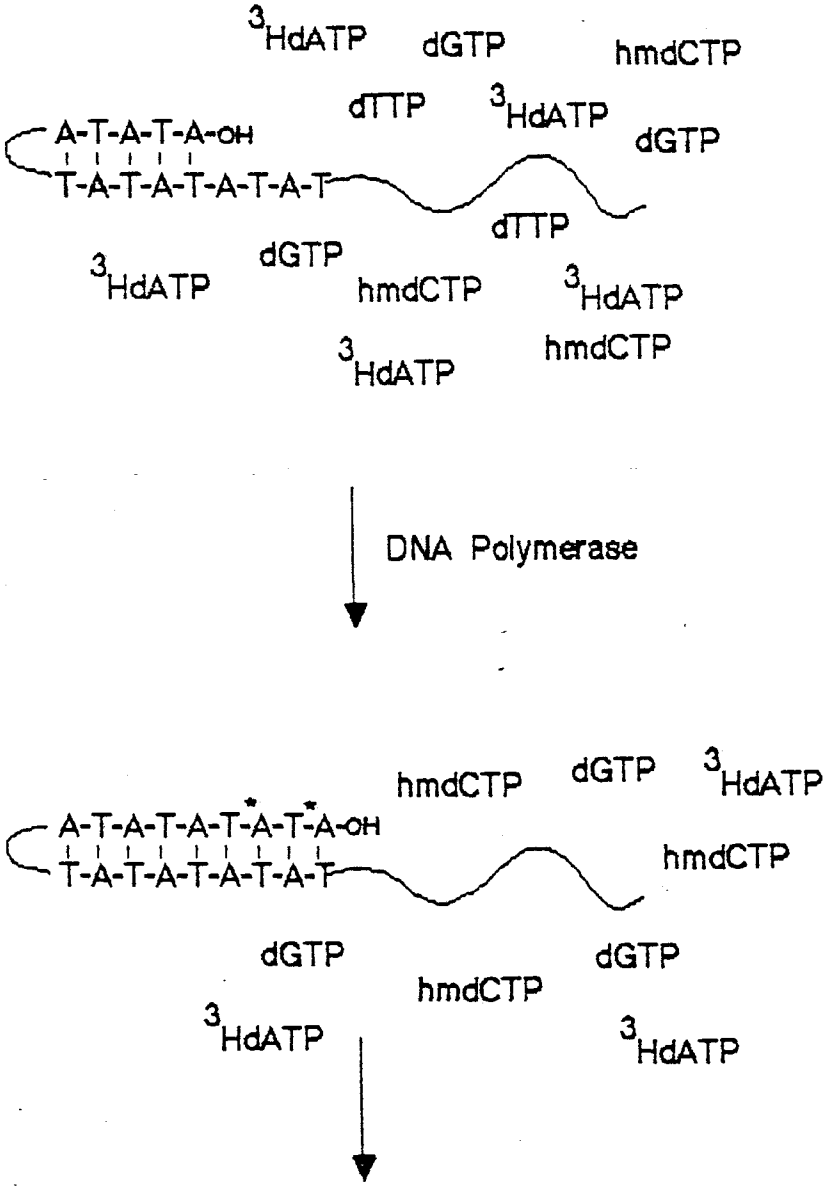


Figure 7. The Enzymatic Assay for dNTPs.

from which to select interesting rII mutants to study. Second, rII^- to rII^+ reversion rarely occurs by extragenic suppression. That is, most rII^- to rII^+ reversion can be shown to occur at, or extremely close to, the original mutant rII site. Third, as described in more detail below, a portion of the rIIB protein is completely disposable for rII function. This provides a region in the rIIB protein that should tolerate any amino acid substitutions, preventing selection for, or against, any base changes leading to potentially incompatible amino acids in the rIIB protein. Finally, even though there is evidence suggesting interaction between the rII proteins and phage replication proteins, there is no evidence that mutations in rII actually affect phage replication or mutation rates.

Mutation rates of AT-to-GC and GC-to-AT transitions, and frameshift mutations, were measured by following rII^- to rII^+ reversion of well-defined rII mutants. These rII mutations are mapped into either of two cistrons, rIIA or rIIB, characterized with respect to the nature of the mutational defect (deletion, frameshift, missense or nonsense mutant), and the putative mutational pathway identified for reversion (transitions, transversions and frameshifts) back to a rII^+ phenotype. Furthermore, spontaneous, chemically, and genetically induced mutation rates have been measured at many sites for forward (rII^+-to-rII^-) and back (rII^--to-rII^+) mutations.

The rII^- and rII^+ phage can be distinguished simply by the difference in phage growth on two different cell lines. Phage mutant in rII do not grow on cells lysogenic for phage λ, but make their characteristic large-plaque morphology on non-lysogenic cells; while rII^+ phage grow on both lysogenic and non-lysogenic E. coli. A

typical test for mutagenesis using this system is to grow *rII*$^-$ phage on non-lysogenic *E. coli* for several rounds of infection, and then determine phage titers on lysogenic and non-lysogenic cells. Changes in mutation rates are detected by an increase in *rII*$^+$ titer, measured on lysogenic cells, and the mutation rates are expressed as the ratio of *rII*$^+$ phage (titer on lysogenic cells)/*rII*$^-$ phage (titer on non-lysogenic cells).

To fully characterize the base changes observed genetically in the *rII* genes, the nucleotide sequence of *rII*$^+$ revertants were determined. As described above, hmdCMP in T4 DNA prevents cleavage by most restriction endonucleases and, hence, precludes sequencing *rII*$^+$ revertants by conventional methods that use cloning as a first step to prepare nucleic acid templates. Furthermore, since I wanted to use sequencing as an analytical tool, the methodology needed to be adapted to recovering and sequencing 10 to 100 templates in a reasonable amount of time. This places the constraint that template preparation and sequencing should require as few manipulations as possible, not only for speedy results, but also to prevent introduction of artifactual mutations in the nucleic acid sequences either during passage through host/vector systems, or chemically, for example, by deamination of template cytosine residues to uracil. A method that meets these requirements is the direct sequencing of T4 messenger RNA, purified from phage-infected cells. Figure 8 outlines the basic procedure. The total complement of low-molecular-weight nucleic acids are purified from phage infected cells. A [^{32}P] end-labelled oligonucleotide primer, complementary to the RNA that will eventually serve as the sequencing template, is added to the heterogeneous RNA

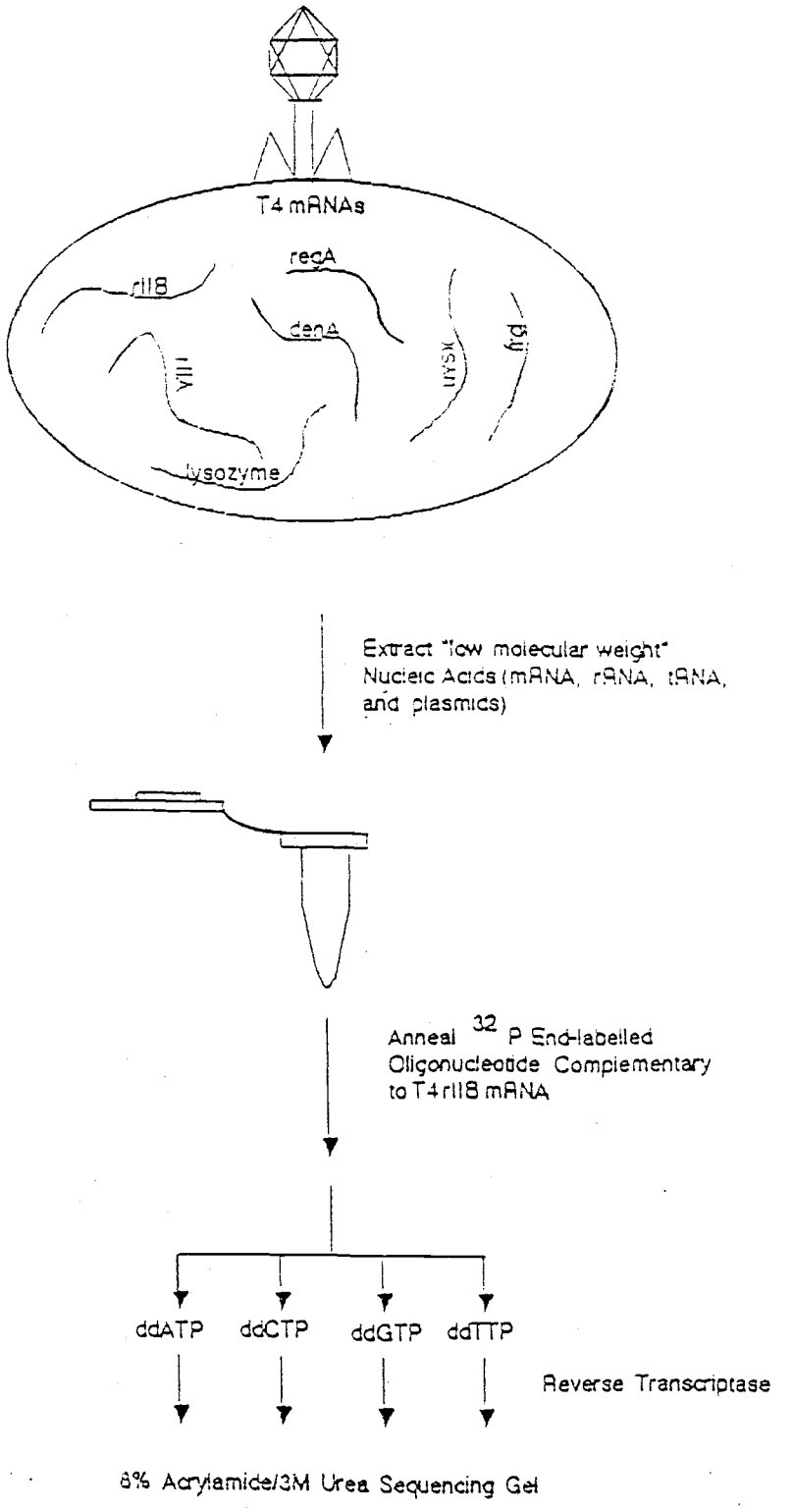


Figure 8. Dideoxy RNA Sequencing.

mixture under conditions that will promote annealing of the primer to the target RNA. One prerequisite for this method is that the wild type sequence of the target RNA is known, hence the primer can be designed to anneal near the site to be sequenced. The primer-bound RNAs, and RNAs to which no primer is bound, are added to sequencing reactions containing AMV reverse transcriptase, the four dNTPs needed for reverse transcription, and a dideoxyribonucleoside triphosphate. After the sequencing reaction has gone to completion, the sequencing-reaction products can be visualized and read similar to conventional dideoxy sequencing reactions.

Some of this work, that described in the chapter on Deoxyribonucleotide Pool Imbalances and Altered Spontaneous Mutation Rates During Infections by T4 dCMP Deaminase Mutants, has been accepted for publication in the Journal of Biological Chemistry.

II. MATERIALS AND METHODS

A. Materials

[^{3}H] Deoxyribonucleoside triphosphates used for dNTP assays were from ICN, while [γ-^{32}P] rATP, for end-labelling of oligonucleotides used in primer extension sequencing of RNA, was from New England Nuclear.

Deoxyribonucleoside and dideoxyribonucleoside triphosphates used for primer extension sequencing were of the highest quality available from PL-Pharmacia, and other deoxyribonucleotides, ribonucleotides, deoxyribonucleosides, and ribonucleosides were purchased from Sigma. BrUdR and hydroxyurea were from Calbiochem. Tri-n-octylamine was from ICN K+K Biochemicals or Aldrich, and Freon-113 was from Matheson.

E. coli DNA polymerase I was from Worthington Biochemicals or the Endonuclease-Free grade from Boehringer-Mannheim. AMV reverse transcriptase was the cruder grade available from Life Sciences. Polynucleotide kinase was from American Biochemical or New England Biolabs. Restriction endonucleases were from New England Biolabs or BRL and used as specified by the vendor.

Primers used for primer extension sequencing of RNA were a gift from the lab of Dr. L. Gold, University of Colorado-Boulder, or synthesized by Dr. R. McParland of the Center for Gene Research and Biotechnology, Oregon State University, and used as supplied without further purification.

B. Media

Nutrient broth contained 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.

Hershey soft agar for phage plating contained 4 g nutrient broth, 5 g NaCl, and 7 g of Difco Bacto-agar per liter.

L-broth had 10 g of Difco Bacto-tryptone, 5 g Difco Yeast Extract, 10 g NaCl, and 1 g glucose per liter. After mixing, the pH was adjusted to pH 7.2 (with pH papers) before autoclaving. L-broth plates contained 12 g Bacto-agar per liter of L-broth, and L-broth soft agar used for phage plating contained 7 g of Bacto-agar per liter L-broth.

M9 medium contained 1 g NH_4Cl, 6 g Na_2HPO_4, 3 g KH_2PO_4, 1 g NaCl, 0.01 g $CaCl_2$, 0.2 g $MgSO_4$-$7H_2O$, and 3 g glucose per liter. SM9 was M9 medium with 2 g vitamin-free Casamino acids per liter.

GPTG medium (Hall, 1967) contained 5.8 g NaCl, 3.7 g KCl, 0.15 g $CaCl_2$-$2H_2O$, 0.10 g $MgCl_2$-$6H_2O$, 1.1 g NH_4Cl, 10 ml of 1.0 M Tris-Cl, pH 7.4, 0.322 g Na_2SO_4-$10H_2O$, 0.10 g β-glycerophosphoric acid disodium salt, 5 g glucose and 1 g vitamin-free Casamino acids per liter. GPTG plates were GPTG medium with the addition of L-tryptophan to 20 μg/ml and 12 Bacto-agar per liter. GPTG soft agar contained GPTG medium with 7 g Bacto-agar per liter.

C. Cells and Phage

Cells and phage used in these studies are summarized in Table III,

Table III. Cells and Phage

Cells		Relevant Phenotype or Genotype	Source
E. coli	B	Wild Type	Our collection
	BB	Suppresses *rII* mutations, resulting in *rII*$^+$ growth and plaques	J. Drake and our collection
	OK305	Defective in *de novo* pyrimidine metabolism and cytidine/ deoxycytidine deaminase	D. Hall
	K38λ	Lysogenic for phage lambda, restrictive host for *rII* phage	D. Pribnow
	CR63	*sup D* amber supressor	Our collection
	CTr5X	Cal Tech hospital strain of *E. coli*, restrictive host for *pseT* T4	Our collection
	ED8689	*sup*^{0}, *hsdR*$^-$, *hsdM*$^+$	D. Hall
	pPS2	*E. coli* HB101 carrying a pBR322-derived plasmid containing both the *nrdA* and *nrdB* genes of *E. coli*	B. M. Sjöberg

Table III. Continued

MB61.21	*E. coli* HB101(λC^+), carrying the pNY10.21 plasmid, constitutively expressing a *Bacillus subtilis*-related thymidylate synthase	M. Belfort
MB61.22	*E. coli* HB101(λC^+), carrying the pNY10.22 plasmid, constitutively expressing a *Bacillus subtilis*-related thymidylate synthase	M. Belfort

Phage	Relevant Phenotype or Genotype	Source
T4D	Wild Type	Our collection
pseT$_\Delta$4	Δ*cd*, Δ*pseT*	D. Hall
pseT$_\Delta$5	*cd*$^+$, Δ*pseT*	D. Hall
cdN16	*cd*$^-$ (missense), *pseT*$^+$	D. Hall
nrdA18	*nrdA*$^-$ (missense)	D. Hall
nrdAam67	*nrdA*$^-$ (amber)	D. Hall
nrdBamB55	*nrdB*$^-$ (amber)	D. Hall
tsJW5	*1*$^-$(dNMP kinase)	Our collection
tsL81	*42*$^-$(dCMP hydroxymethyl-transferase)	Our collection
tsLB342$^-$		Our collection
rIIUV215	*rIIA*$^-$	J. Drake
rIIamHB84	*rIIA*$^-$ (amber)	D. Pribnow
rIIHD263	*rIIB*$^-$	L. Gold
rIIamHB74	*rIIB*$^-$ (amber)	D. Pribnow
rIISN103	*rIIB*$^-$	Our collection
rIIUV363	*rIIB*$^-$	J. Drake

and their construction and propagation are described in the text.

D. Preparation of Cell Extracts for dNTP measurements

Overnight cultures of E. coli grown in nutrient broth or SM9 were diluted 1:100 into nutrient broth or SM9 and grown with aeration at 37°C to 3 x 10^8 cells per ml. Immediately before infection, L-tryptophan was added to 20 µg/ml and a sample of cells plated in duplicate to determine cell titer. Phage were added at 5 phage per cell, and 50 ml of phage-infected E. coli were harvested at 5-minute intervals, by rapid filtration through stacked glass-fiber prefilters (Payne and Ames, 1982). The filters were quickly immersed in either cold (4°C) 60% methanol/1% toluene for four hours or cold 5% trichloroacetic acid for one to two hours and a known concentration of xanthosine triphosphate added as an external marker to assay for recovery of nucleotides.

60% methanol/1% toluene extracts were taken to dryness by lyophilization or by a Savant Speed Vac concentrator. The residue was resuspended in 2 ml of cold 5% trichloroacetic acid and spun at 10,000 x g, 4°C, for 30 minutes and the supernatant saved. The 5% trichloroacetic extracts were spun at 10,000 x g, 4°C, for 30 minutes and the supernatant saved.

Extracts that originated from 60% methanol/1% toluene or 5% trichloroacetic acid-extracted filters were processed identically from this point on. The trichloroacetic acid was removed by extraction of the 5% trichloroacetic acid supernatants from above, with 1.5 volumes of 0.5 M tri-n-octylamine in Freon (Chen et al., 1977; Khym, 1975).

The aqueous layer (pH 5.5 to 6.5) was recovered, taken to dryness by lyophilization or by a Savant Speed Vac concentrator and the residue dissolved in a minimal volume of water (usually 0.25 ml).

E. The Enzymatic dNTP Assay

Deoxyribonucleoside triphosphate concentrations of cell extracts were determined essentially as described by North et al. (1980) with some modifications. The concentrations of deoxyribonucleoside triphosphates and ribonucleoside triphosphates, used as standards, were determined spectrophotometrically, assayed for purity by HPLC and the (deoxy)ribonucleotide concentrations corrected for contaminating (d)NMPs and (d)NDPs. The reaction mixtures (100 µl final volume) for the dNTP assays were 45 mM Tris-Cl, pH 8.3, 4.5 mM $MgCl_2$, 1 mM β-mercaptoethanol, 1 µM[^{3}H]dNTP (10 mCi/µmol) complementary to the dNTP being assayed, 1 unit/ml *E. coli* DNA polymerase I (endonuclease free), 20 µM template (ca. 8 µg/ml), 0.2 mg/ml bovine serum albumin, 10 mM dAMP, 10 µl of dNTP standard or cell extract. Reaction mixtures routinely contained 10 mM dAMP to inhibit the 3'-to-5' exonuclease activity of *E. coli* DNA polymerase I and prevent template degradation (Hunting and Henderson, 1981).

The assay components were mixed at 4°C, incubated at 37°C, and 25-µl samples (taken at 75 min, 90 min and 105 min) pipetted onto Whatman 3 MM chromatography paper squares presoaked with 5% trichloroacetic acid/2% sodium pyrophosphate. The squares were washed by ascending chromatography with 5% trichloroacetic acid/1% sodium pyrophosphate and washed twice for 15 minutes per wash in 95% ethanol

(Weinstein et al., 1975) before scintillation counting.

F. HPLC of Nucleotides

Nucleotide concentrations and purity were determined by high performance liquid chromatography on a Varian 5000 HPLC. Samples were injected onto a 4-mm x 25-cm column packed with the strong anion exchanger Partisil-10 SAX (Whatman), eluted with 0.35 M potassium phosphate buffer, pH 3.8, and detected by absorbance at 254 nm. A Labtronix autosampler was used for automated injection of samples. Data were collected and processed on a Varian CDS 401 computer.

G. T4 dCTPase/dUTPase Purification

Purification of T4-induced dCTPase/dUTPase was done essentially as described by Warner and Barnes (1966). Streptomycin sulfate (2% w/v) precipitates from bacteriophage T4 infected E. coli (supplied by Gerry Lasser of our laboratory) were stored at -20°C in an equal volume of glycerol until used. 25 ml of 0.4 M potassium phosphate, pH 6.9, 2 mM β-mercaptoethanol, was added to 25 ml of streptomycin sulfate pellet in glycerol and allowed to resuspend overnight at 4°C. The resuspended pellets were centrifuged at 12,500 x g for 60 minutes and the supernatant diluted with 0.01 M potassium phosphate, pH 6.9, 2 mM β-mercaptoethanol, to give a solution of 0.05 M potassium phosphate, 2 mM β-mercaptoethanol.

This solution was loaded onto a 2.5-cm x 40-cm DEAE-cellulose column, preequilibrated with 0.01 M potassium phosphate, 2 mM

β-mercaptoethanol, at 1-2 ml per minute and washed with 0.05 M potassium phosphate, 2 mM β-mercaptoethanol until the A_{280} stabilized. The dCTPase/dUTPase activity was eluted with a linear NaCl gradient with a flow rate of 1-2 ml per minute. The mixing chamber contained 500 ml of 0.01 M potassium phosphate, 2 mM β-mercaptoethanol, and the reservoir contained 500 ml of 0.01 M potassium phosphate, 2 mM β-mercaptoethanol, 0.7 M NaCl. 10-ml fractions were collected and assayed for dCTPase/dUTPase activity. Fractions containing dCTPase/dUTPase activity were stored in 50% glycerol at -20°C.

H. Enzyme Assays

T4 dCTPase/dUTPase activity was assayed by following the cleavage of [^{3}H]dCTP to [^{3}H]dCMP, using the reaction conditions described by Warner and Barnes (1966) and separation of the reactants by anion exchange (polyethyleneimine-cellulose) thin layer chromatography. Reaction mixtures contained 25 μM $MgCl_2$ 40 μM Tris-Cl, pH 8.5, 10 μM β-mercaptoethanol, 1 μM EDTA, 1 mM [^{3}H]dNTP (>10 μCi/μmole) and enzyme. Reactions were started on ice, incubated at 37°C and stopped by adding an equal volume of reaction mix to 10% trichloroacetic acid containing deoxyribonucleotide standards. 10 μl of the acid-stopped reaction was spotted onto a sheet of polyethyleneimine-cellulose (Brinkmann) and desalted by washing 30 minutes in 100% methanol. The sheets were dried at room temperature and developed by ascending chromatography in 1.5 M LiCl. After the solvent front had reached the top of the TLC sheet, the sheets were removed from the solvent tank, desalted by washing in an excess of 100% methanol for at least 30

minutes and dried at room temperature. Deoxyribonucleotide spots were visualized by incident ultraviolet light, cut out from TLC sheets and eluted for 30 minutes in a scintillation vial with 0.5 ml of 0.5 N HCl. 5 ml of a Triton X-100-based scintillation cocktail was added and the samples counted.

I. Protein Determinations

Protein concentrations were determined by Coomassie Blue dye binding (Peterson, 1983). Dye stock solutions were made by dissolving 1 gm of Coomassie Brilliant Blue G-250 (Eastman Kodak) in 200 ml of 88% phosphoric acid and 100 ml of 95% ethanol. 150 ml of the stock solution was diluted to 600 ml with water and filtered through Whatman No. 1 filter paper. 10 µl of sample of protein standard (BSA) were added to 1.0 ml of water followed by 1.5 ml of filtered dye solution. The absorbance (595 nm) of the samples was read after a 5-to-10 minute incubation at room temperature.

J. Phage Crosses

Crosses between rII mutants and phage mutants in genes involved in T4 deoxyribonucleotide metabolism were done as described by Hall et al. (1967). One to two drops of each mutant phage were added to one drop of fresh E. coli, at about 5 x 10^8 cells/ml. The final multiplicity of phage per cell in the crosses was 5:1 to 10:1. Usually a 10:1 ratio of phage mutant in deoxyribonucleotide metabolism to rII mutant phage was used in the crosses to increase the chance of

finding doubly mutant phage amongst rII mutant phage recovered.

Crosses using T4 amber mutants were performed on E. coli CR63, a supD amber suppressor cell line, and all other crosses were done on the non-suppressor cell E. coli B. The infected cultures were incubated one hour at 30°C for crosses using T4 temperature sensitive mutants and 37°C for all other crosses. Infections were terminated by adding a few drops of chloroform to the infected cells and allowing 5 to 10 minutes for the phage infected cultures to complete lysis. These lysates were diluted and plated on E. coli B or E. coli CR63. Isolated rII plaques were picked into two ml of nutrient broth containing a few drops of chloroform, and these stocks were for further screening.

T4 mutant in the rII genes do not plate E. coli lysogenic for λ phage, hence, phage carrying rII mutant alleles were identified by spotting single plaque isolates of T4 crosses on lawns seeded with E. coli K38λ cells. T4 phage carrying either deletion mutant pseTΔ4(Δcd, ΔpseT) or pseTΔ5(cd$^+$, ΔpseT) were identified by plating on E. coli CTR5x. This cell line restricts growth of phage mutant in the T4 gene encoding polynucleotide kinase (pseT).

T4 mutant in the genes encoding ribonucleotide reductase or the gene encoding dCMP deaminase normally do not have strong restrictive conditions for phage growth; thus identification of phage mutant in these genes can be difficult. However, phage mutant in these genes can be identified by their ability to suppress the so-called "halo" plaque phenotype when plated on the E. coli host cell OK305, in the presence of the folate analog pyrimethamine (see below). To identify rII x dCMP deaminase or rII x ribonucleotide reductase doubly mutant

phage produced by the crosses, single plaque isolates were diluted 10,000-fold into GPTG medium and plated under "halo"-producing conditions to score for the ability of isolates to suppress the "halo" plaque phenotype.

Those phage isolates identified as doubly mutant by spot tests were plaque purified once more and tested again under the appropriate plating conditions to identify the presence of rII, and the other, mutant alleles and used as the master stock for reversion tests and production of lysates.

K. Halo Plaque Plating

Halo plaques are actually rings of dense cell growth surrounding a phage plaque, fed by nutrients released from T4-lysed cells. This phenomenon relys on defects in the host cell nucleotide metabolism, and the blockage of T4 thymidylate synthesis by an antimetabolite, pyrimethamine, directed against the phage dihydrofolate reductase. E. coli OK305 cells are defective in de novo UMP biosynthesis, as well as containing only a fraction of their normal cytidine/deoxycytidine deaminase activity. These cells grow well in minimal medium containing uracil as a source of pyrimidine nucleosides, but grow at an extremely slow rate when grown in the presence of cytidine. When phage are plated on these cells in the presence of cytidine and pyrimethamine, the block imposed on T4 thymidylate synthesis is believed to cause accumulations of intracellular dUMP, deoxyuridine, and uracil. On lysis of the infected cells these nutrients are released into the surrounding medium, and supply a locally high

concentration of nucleotides, nucleosides, and bases for cell growth. Mutations in deoxyribonucleotide metabolism upstream of T4 thymidylate synthase, for example, in phage dCMP deaminase or ribonucleotide reductase, will prevent or severely limit the biosynthesis of dUMP and thwart halo plaque formation.

E. coli OK305 cells were grown in GPTG supplemented with 20 µg/ml uracil to stationary phase (about 2 x 10^9 cells per ml) and centrifuged at 6,000 x g, 4°C, for 10 minutes. The cells were resuspended in 1/10 volume of cold GPTG (no uracil) and kept on ice until used. To induce halo plaques with wild type or rII T4, about 2 x 10^9 cells (0.1 ml of 10 x concentrated cells) were added to GPTG soft agar that contained 800 µg of the folate analog pyrimethamine (0.2 ml of 4 mg/ml pyrimethamine) and 300 phage (Johnson and Hall, 1973). This mixture was plated on GPTG plates containing 20 µg/ml cytidine and incubated for 8 to 12 hours at 30°C. If halo plaques were not visible by this time, the plates were incubated at 37°C until plaques could be seen. It is very important to plate both a positive (wild type or rII) halo-forming control and negative (nrd or cd) non-halo-forming control for comparison of plaque phenotypes. It is not uncommon for the halos to be weak, or possibly in the case of leaky T4 mutants, to get an intermediate halo phenotype.

L. Reversion Tests

Measurement of mutation rates in T4-infected E. coli were done essentially as described by Drake (1970). Stocks of E. coli B or BB cells grown overnight in nutrient broth or SM9 were used to start

daily cultures. From a 1:50 dilution of overnight culture into nutrient broth or SM9, cells were grown to a density of 2 to 5 x 10^8 cells/ml and diluted, into prewarmed medium containing 20 µg/ml L-tryptophan, to a density of 2 x 10^7 cells/ml. Ten to 100 phage were added to 5-ml portions of diluted cell culture and the infections allowed to proceed for 3 hours with aeration. A few drops of chloroform were added to stop the infections followed by 10 to 15 minutes' further incubation to allow lysis of any remaining infected cells. Lysates were diluted into nutrient broth and plated on nutrient broth plates containing the permissive host E. coli B cells for lysate titers and on the non-permissive host for rII mutant phage, E. coli K38λ cells, to determine the titer of rII$^+$ revertants.

M. RNA Purification

The nucleic acid sequences of rIIamHB74$^+$ revertants were determined by dideoxy sequencing, using T4 rIIB messenger RNA as template. This technique was described by Zaug et al. (1984) for sequencing Tetrahymena ribosomal RNA and adapted by D. McPheeters and M. Inman in the laboratory of Dr. L. Gold for sequencing bacteriophage T4 messenger RNA (McPheeters et al., 1986).

E. coli B or NAPIV cells grown overnight in M9 were diluted 1:50 into fresh M9 and grown at 30°C with aeration to a titer of 3 x 10^8 cells/ml. L-tryptophan was added to 20 µg/ml and the cells divided into 10 ml cultures. These cultures were infected with rIIamHB74$^+$, pseTΔ4/rIIamHB74$^+$, or pseTΔ5/rIIamHB74$^+$ revertants (10 phage/cell) and aerated at 30°C. At 10-12 minutes post-infection, the phage

infections were terminated by decanting the cultures into chilled Corex centrifuge tubes and incubating in an ice-water bath for 10 to 15 minutes. The chilled cultures were spun for 15 minutes at 10,000 x g, 4°C, and each supernatant decanted. The pellets were resuspended in 0.225 ml of cold, sterile 0.5% NaCl and transferred to chilled, sterile 1.5 ml microcentrifuge tubes.

From this point on, all solutions and plasticware were prepared and handled with the utmost care in order to prevent introduction of exogenous RNAses and the enzymatic degradation of RNA.

25 µl of 10X lysing buffer (0.5 M Tris, pH 6.8; 20 mM EDTA; 10% SDS) was added to the tubes containing the resuspended pellets, and the tubes incubated at 68°C for 3 minutes. 50 µl of 1.0 M sodium acetate, pH 5.2, was immediately added followed by mixing. These solutions were extracted 3 times with 50% phenol/48% $CHCl_3$/2% isoamyl alcohol.

The nucleic acids were precipitated by addition of 1/10 volume of 5 M ammonium acetate followed by 2.5 volumes of cold (-20°C) 95% ethanol and incubation for at least two hours at -20°C. Ethanol precipitates were collected by centrifugation fo 30 to 40 minutes at 12,000 x g, 4°C, in an Eppendorf centrifuge, the 95% ethanol supernatant discarded and the pellets washed once with cold (-20°C) 70% ethanol and recentrifuged for 5 minutes, 4°C. The 70% supernatant was discarded, the nucleic acid pellets dried <u>in vacuo</u> and resuspended in 30 µl of 1 mM EDTA. These preparations typically had an A_{260}/ml of 100 and A_{260}/A_{280} ratios of 2. The resuspended nucleic acid pellets were stored at -20°C until used.

N. RNA Primer-Extension Sequencing

Oligonucleotides used for primer-extension sequencing were 5'-end-labeled as described by Maniatis et al. (1982). Since the end-labelled oligonucleotide primers were to be used in sequencing reactions using RNA as template, all plasticware and reagents were kept as free of exogenous RNAse contamination as possible. Reaction mixtures contained: 50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM spermidine, 0.1 mM EDTA, 10 pmole of oligonucleotide primer, 10 pmole of [γ-^{32}P]ATP (3000 Ci/mmol) and 10 units of T4 polynucleotide kinase. End-labeling reaction mixtures were incubated at 37°C for 45 minutes and then shifted to 95°C for 3 minutes to stop the reaction. Tne end-labeled primers were extracted once with 50% phenol/48% $CHCl_3$/2% isoamyl alcohol and once with water-saturated diethyl ether. The aqueous phase, containing the labelled oligonucleotides, was dried in a Savant Speed Vac concentrator and resuspended in 20 µl of sterile water.

Annealing reactions of primers to RNA templates contained 50 mM Tris-Cl, pH 8.6, 60 mM NaCl, 10 mM dithiothreitol, 0.1 to 0.2 pmol of nucleic acid template (8 to 10 µl of resuspended cell RNA) and 0.3 to 0.6 pmol end-labeled primer. The reactions were heated at 60°C for 3 minutes, quick-chilled in dry ice/ethanol for one minute and placed on ice until used.

Sequencing reaction mixtures (5 µl) contained 50 mM Tris-Cl, pH 8.6 60 mM NaCl, 10 mM dithiothreitol, 30 mM magnesium acetate, 400 µM dTTP, 400 µM dATP, 400 µM dCTP 400 µM dGTP, 50 µM dideoxynucleoside triphosphate (ddATP, ddTTP, ddCTP, or ddGTP), 2 µl of the annealing

reaction, and 0.7 units of AMV reverse transcriptase. Reaction mixtures were incubated at 48°C for 30 minutes and the reactions stopped by chilling on ice and adding 5 µl of 2X Loading Dye [94% formamide, 4% Tris-borate-EDTA (see below), 0.04% Bromphenol Blue, 0.04% Xylene Cyanol].

The sequencing reactions were heated for 3 minutes at 95°C immediately prior to electrophoresis and 8 to 10 µl of each sequencing reaction loaded on an 8% (38:2 acrylamide:bis acrylamide), 7 M urea, sequencing gel in Tris-borate-EDTA buffer (0.089 M Tris-borate, 0.089 M Borate, 0.02 M EDTA). The gels were run at approximately 1500 volts DC, constant voltage (this corresponded to a running temperature of about 50 to 60°C) for 4 hours, at which time the gels were wrapped in Saranwrap and radioautographed with Kodak X-Omat XAR-5 or SB-5 film at -70°C for 3 days to 2 weeks.

O. Construction and Propagation of E. coli ED8689/pBR322 and ED8689/pPS2

To construct a sup° cell line overproducing E. coli ribonucleotide reductase, the plasmid pPS2, which contains both subunits of E. coli reductase, was transferred from its original host, E. coli HB101 (hsdr$^-$, hsdm$^-$, supE44), into E. coli ED8689 (hsdr$^-$, hsdm$^+$, sup°). This plasmid was constructed by Platz and Sjöberg (1980) by cloning a Pst 1 fragment from E. coli, containing both the nrdA and nrdB genes, into the Pst 1 site of pBR322. Cells carrying pPS2 were identified by their resistance to tetracycline (12.5 µg/ml), hydroxyurea (5 mg/ml) and sensitivity to ampicilliln (50 µg/ml). Plasmid purification and

cell transformations were according to Davis et al. (1980). To ascertain that tet^r, HU^r, amp^s ED8689 cells were in fact carrying pPS2, restriction digests of plasmid DNA from these cells were compared to 1% agarose patterns of authentic pPS2 DNA (Platz and Sjöberg, 1980).

For experiments requiring growth of ED8689/pPS2 or ED8689/pBR322 in liquid medium, cells were grown in the presence of tetracycline alone. With regard to hydroxyurea sensitivity, this phenotype was best determined on solid medium, as some growth was always observed for HU^s cells in liquid medium containing hydroxyurea.

P. Phage Bursts

Cells from a culture grown overnight were diluted 100-fold into fresh medium and grown with aeration to 2 to 5 x 10^8 cells/ml. L-tryptophan was added to 20 µg/ml and the cells infected with phage (0.1 phage/cell). After a two minute incubation, to allow adsorption of phage to the cells, the cultures were diluted 10^6-fold into prewarmed medium, and incubated with aeration. At this time, a parallel 10^6-fold set of dilutions were constructed, except that the dilution medium contained a few drops of chloroform to halt the progress of phage growth in infected cells. Both of these cultures were plated in duplicate on permissive cells to carefully determine the titer of infecting phage (unchloroformed dilutions) and the titer of unadsorbed phage left in the culture (chloroformed dilutions).

At 30- to 90-minutes post-infection, chloroform was added to the incubating phage-infected cell cultures, and several minutes further

incubation to allowed for lysis of phage-infected cells. Phage titers were determined by duplicate platings of these lysates on permissive cells. Accurate measurements of phage produced during infections required the fraction of unadsorbed phage to total phage added at the beginning of the experiment to be 5% or less. Phage burst sizes were calculated by the ratio of the phage titer at the end of the incubation period (30- to 90-minutes post-infection) to the number of infected cells (total phage added at the beginning of the experiment less unadsorbed phage).

III. RESULTS

In principle, the experimental strategy used in these studies was straightforward. I altered intracellular nucleotide pools through mutations in deoxyribonucleotide metabolism, or by addition of deoxyribonucleosides to the growth medium. The consequences of these imbalanced dNTP pools, in terms of replication fidelity, were determined indirectly, by measuring mutation rates at well-defined sites in the T4-rII genes. Finally, the actual mutagenic event, responsible for changes in mutation rates, was identified by nucleic acid sequencing through the site in the rII messenger RNA.

A. Artifacts of Nucleotide Extraction and Measurement

Probably the most tedious and problematic technology used in these studies was the extraction and measurement of intracellular deoxyribonucleotides. Sample preparation necessarily requires rapid harvesting of cells and the quantitative extraction and recovery of nucleotides during processing of cell extracts, while minimizing nucleotide degration. Second, since the assay used to measure dNTP concentrations in cell samples is an enzymatic assay, using DNA polymerase, it is important to not introduce reagents or activities, either during cell treatments or sample preparation, that will interfere with the assay.

Two methods for extraction of nucleotides from harvested, phage-infected cells were used: 60% methanol/1% toluene extraction of cells at 4°C for 4 hours, followed by lyophilization, treatment with

5% trichloroacetic acid, extraction of the trichloroacetic acid out of the aqueous phase with tri-n-octylamine/Freon, lyophilization and resuspension of the residue in water (MeOH/TCA/TOA extraction); or, cell extraction in 5% trichloroacetic acid at 4°C for 30 min, followed by tri-n-octylamine/Freon extraction, lyophilization, and resuspension of the residue in water (TCA/TOA extraction). Both methods were based on procedures used for extraction of nucleotides from mammalian cells, and have been shown to give quantitative recovery of nucleotides without introducing activities that interfere with the enzymatic dNTP assay (North et al., 1980; Hunting and Henderson, 1982).

However, occasionally during these investigations there were significant variations in the recovery and measurement of (deoxy)ribonucleotides from MeOH/TCA/TOA extracts of T4-infected cells. Degradation of nucleotides during sample preparation was particularly evident when methanol/toluene extracts were taken to dryness, prior to trichloroacetic acid treatment, by using a Savant evaporator instead of lyophilization as used for other samples (compare Fig. 10 and Fig. 11). Generally, lyophilization was slightly quicker, and maintained the samples at colder temperatures than the Savant evaporator. I believe that these factors in particular, during lyophilization, helped to minimize enzymatic degradation of nucleotides during this step in sample preparation. North et al. (1980) described similar problems with methanol extracts of mammalian cells, not treated with trichloroacetic acid, and determined that contaminating cell (deoxy)ribonucleotide kinases, coextracted with nucleotides and carried through sample preparation, were enzymatically degrading nucleotides in solution.

The recoveries of nucleotides from rXTP-spiked wild type and tsJW5 infected cells, treated by the methanol/toluene extraction, are summarized in Figure 10. The tsJW5 mutant is not as sensitive to temperature as are other T4 conditional lethal mutants, thus even at 36°C there is phage growth, indicating at least partial dNMP kinase activity (see Table XII). Degradation of all 5 rNTPs, including the rXTP, is seen in extracts made from infections harvested at 5 minute intervals at 36°C (Fig. 9, Fig. 10), while there is little rNTP breakdown seen in extracts made at 40°C (Fig. 9, Fig. 10). This is consistent with the idea that enzymes responsible for nucleotide degradation during sample preparation, might be somewhat less stable in methanol/toluene extracts of 40°C infections, but active or more stable in methanol/toluene extracts made from infected cells grown at lower temperatures.

Likewise, there are activities co-extracted by methanol/toluene treatment capable of deoxyribonucleotide degradation (Table IV). Preincubation of extracts before measuring dNTPs was to allow nucleotide-degrading activities the opportunity to use the available deoxyribonucleotides as substrates. Treatment of the extracts with trichloroacetic acid evidently inactivates this activity, since dTTP measured in incubated trichloroacetic acid-treated extracts was comparable to unincubated MeOH extracts (Table IV).

The recoveries of rXTP from uninfected and infected cells, prepared by the TCA/TOA procedure, are summarized in Figure 11, which shows substantially improved recoveries over samples prepared by methanol/toluene extraction.

Since the intracellular concentrations of rNTPs are roughly 10

Figure 9. HPLC Profiles of Ribonucleoside Triphosphates from Methanol/Toluene Extracts of Temperature-sensitive Gene 1 (dNMP kinase) Infections. E. coli grown in SM9 were infected by a temperature-sensitive gene 1 mutant, tsJW5, at 36°C (Panels a through e), or 40°C (panels f through j); and samples were taken at 0 min (panels a and f), 5 min (panels b and g), 10 min (panels c and h), 15 min (panels d and i), and 20 min (panels e and j) post-infection. Nucleotides were extracted from the phage-infected cells by treatment with methanol/toluene and prepared as described in Materials and Methods. Approximately 500 nmol rXTP was added to the cell extracts, during the methanol/toluene extraction, as a marker to monitor recovery of nucleotides during sample processing. Nucleotides were separated by HPLC of cell extracts (100 µl per injection) and visualized by absorbance at 254 nm. The order of elution was: rCTP ≈ 9.0 min, rUTP ≈ 12 min, rATP ≈ 14 min, rXTP ≈ 18 to 19 min, and rGTP ≈ 26 min.

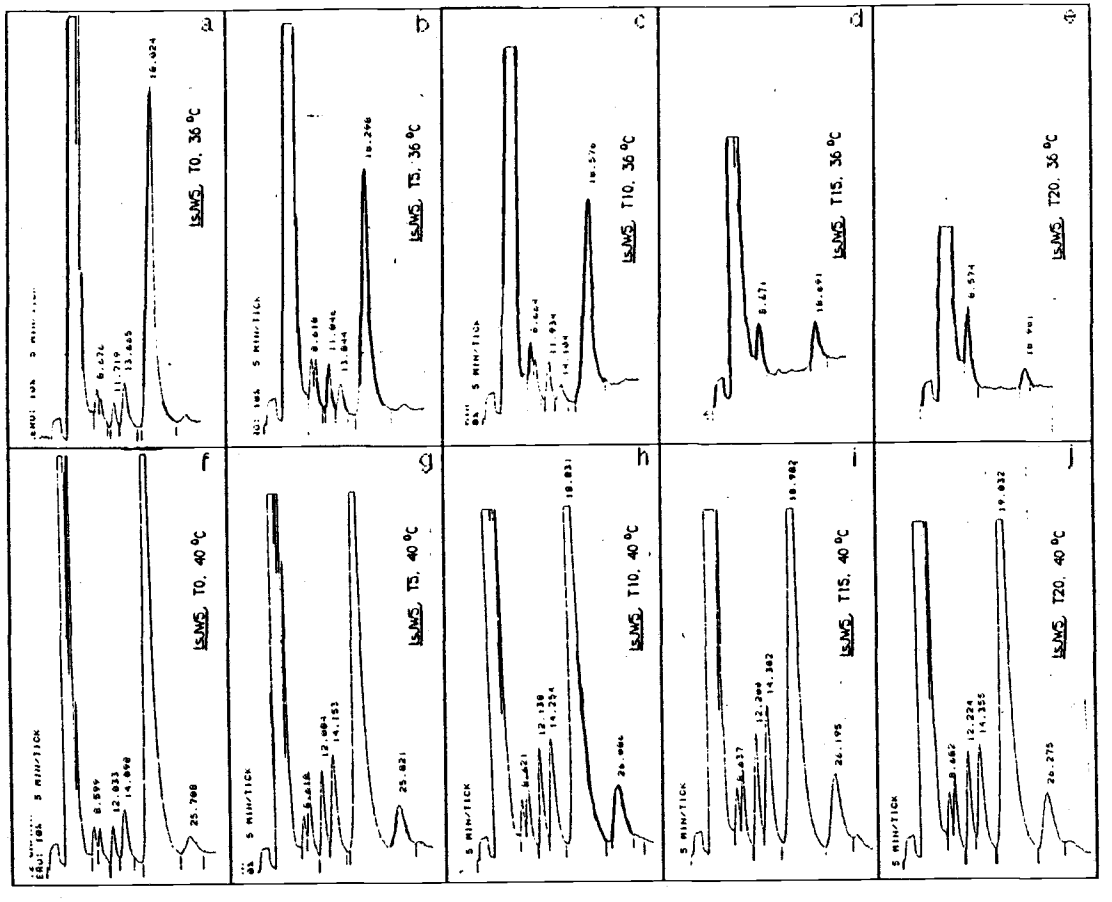


Figure 9. HPLC Profiles of Ribonucleoside Triphosphates from Methanol/Toluene Extracts of Temperature-sensitive Gene 1 (dNMP kinase) Infections.

Figure 10. Recovery of rXTP from Methanol/Toluene Extracts of T4 Wild Type and Temperature-Sensitive Gene 1 (dNMP kinase) Infections. Samples were prepared, and nucleotides measured, as described in Fig. 9. % rXTP recovery is the concentration of rXTP recovered from wild type (panel a) or temperature-sensitive gene 1, tsJW5 (panel b) extracts, divided by the expected rXTP concentration in the processed cell samples.

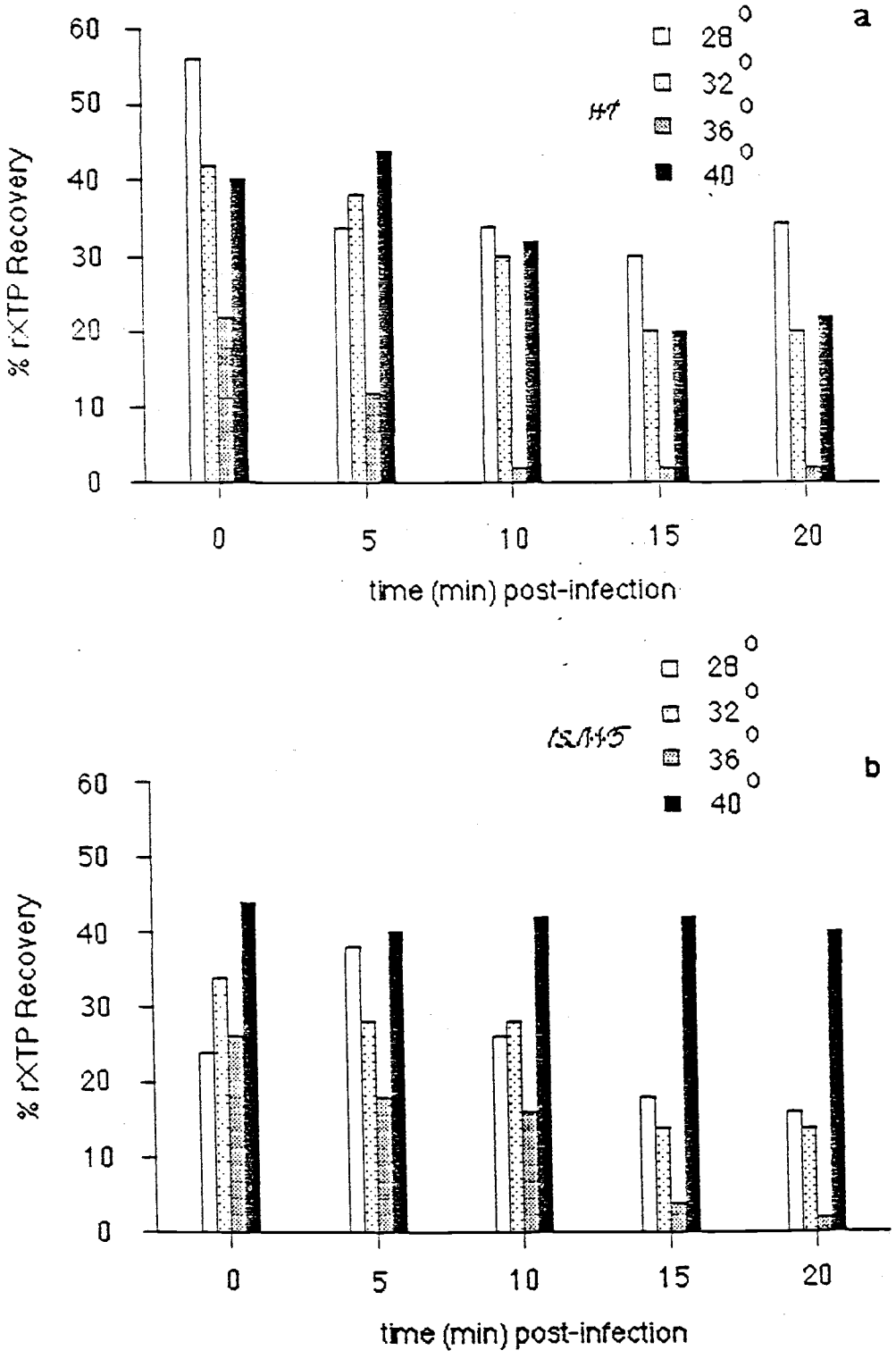


Figure 10. Recovery of rXTP from Methanol/Toluene Extracts of T4 Wild Type and Temperature-Sensitive Gene 1 (dNMP kinase) Infections.

Table IV. dNTPs measured in Samples at Various Stages of Sample Preparation

	pmol dNTP/10 µl		
	Stage of Extraction		
	MeOH/Toluene		MeOH/Toluene/TCA
	A†	B*	C†
dTTP	35	7	23
hmdCTP	10	7	8
dATP	110	28	15
dGTP	7	8	6

† Extracts were not preincubated before measuring dNTPs.

* Extracts were preincubated at 37 °C for 60 min before measuring dNTPs.

Phage-infected cells were harvested at 10 minutes post-infection and nucleotides extracted with methanol/toluene. These extracts were lyophilized, resuspended in 0.25 ml of water, and the dNTP concentrations measured (MeOH/Toluene samples, column A and B). An equal volume of 10% trichloroacetic acid was added to the resuspended MeOH/toluene samples, the trichloroacetic acid extracted with 0.5 M tri-n-octylamine in Freon, the aqueous phase recovered and lyophilized. These samples were redissolved in 0.25 ml of water and the dNTPs measured (MeOH/Toluene/TCA samples, colume C).

times greater than those of dNTPs (Mathews, 1972), a 1000% increase in dNTP concentration from the action of contaminating nucleotide kinases would be seen in only a 10% decrease in rNTP concentration. Even though TCA/TOA extracts overall had improved nucleotide recoveries, this still does not identify whether the 60% rXTP recovery measured in some TCA/TOA extracts (Fig. 11, 15 minute extract), was from pipetting errors, other volume losses, or from nucleotide degradation. Another measurement indicative of nucleotide degradation is the ratio of rATP to rADP present in extracts (Hunting and Henderson, 1982). Figure 12b shows the HPLC chromatogram for the separation of rUDP and rADP for the same wild type extract shown in Figure 12a. Figures 12c and 12d show two rADP standards for comparison. A ratio of rATP:rADP of greater than 10 has been taken (Hunting and Henderson, 1982) to indicate minimal nucleotide degradation during extraction and sample preparation. The rATP/rADP ratio in this extract (Fig. 12) was at least 10, and, in spite of the 60% rXTP recovery measured in this sample (Fig. 11), the high rATP/rADP ratio implies that any loss of nucleotides was due to the physical loss of sample rather than nucleotide degradation.

Historically, nucleotide pools in procaryotes have been measured by labelling intracellular nucleotides to equilibrium with [32P]-orthophosphate of a known specific activity added to the growth medium. The nucleotides, once extracted from the cells, were separated by thin layer chromatography, and spots corresponding to the nucleotides were cut out and counted. One then calculated the nucleotide concentrations from the specific activity of the [^{32}P]-orthophosphate originally added to the medium. This technique

Figure 11. Recovery of rXTP from Trichloroacetic Acid Extracts of Uninfected and T4 Wild Type Infected E. coli. Extracts were prepared essentially as described in Fig. 9, except that nucleotides were extracted with 10% trichloroacetic acid instead of methanol/toluene. Infections were carried out at 37°C, the temperature at which the greatest degradation of nucleotides was observed in methanol/toluene extracts (Fig. 9).

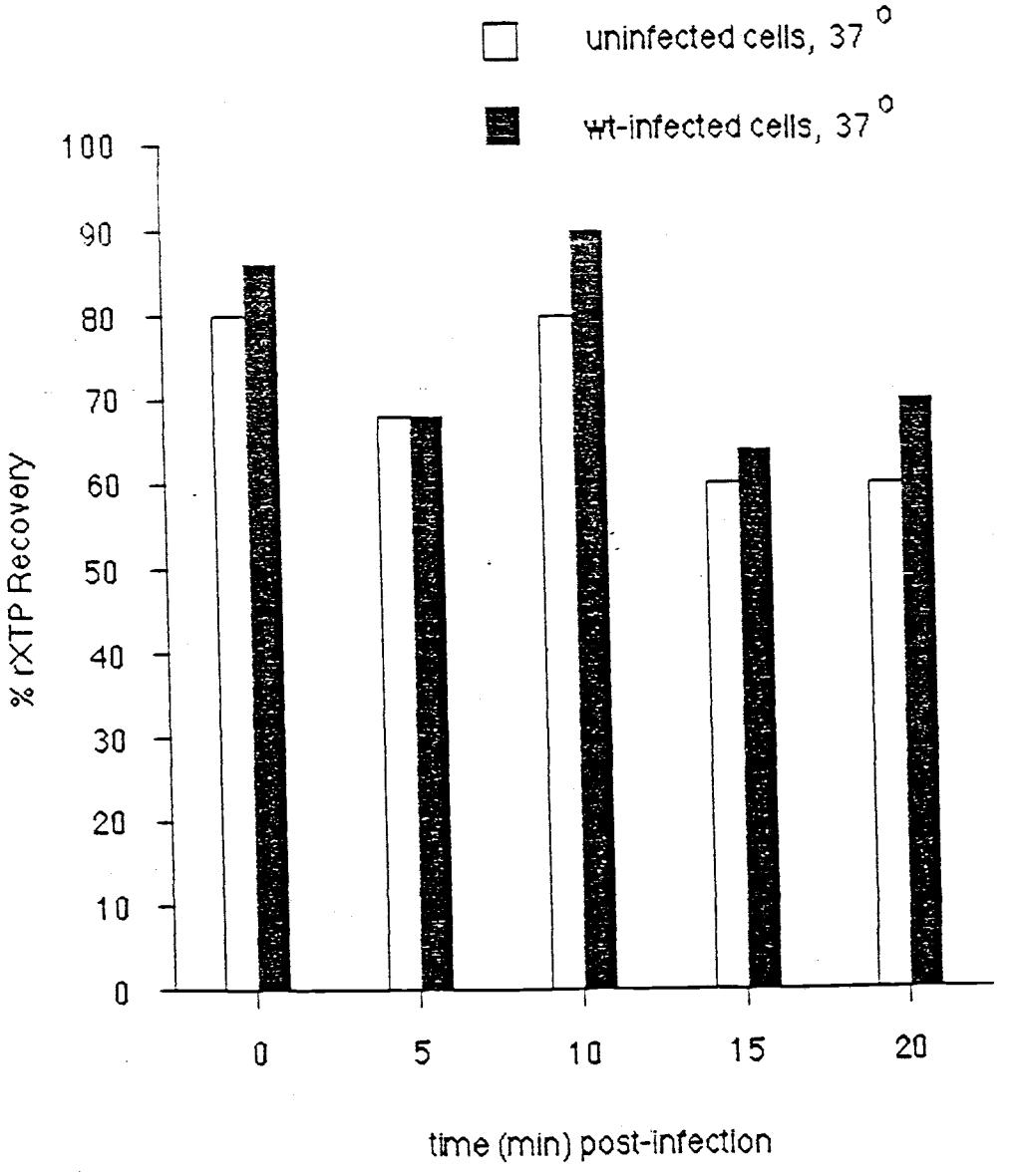


Figure 11. Recovery of rXTP from Trichloroacetic Acid Extracts of Uninfected and T4 Wild Type Infected E. coli.

Figure 12. HPLC Profiles of Ribonucleoside Triphosphates and Ribonucleoside Diphosphates Recovered from Trichloroacetic Acid Extracts of Uninfected and T4 Wild Type Infected E. coli. Extracts were prepared as described in Figure 11 (15 minutes post-infection sample), and Materials and Methods. Ribonucleoside diphosphates were separated on a Partisil-10 SAX column and eluted with 0.050 M potassium phosphate buffer, pH 4.1. The panels are: a) rNTPs from the 15 minute post-infection sample, b) rNDPs from the 15 minute post-infection sample, c) 180 pmol of rADP, d) 90 pmol rADP.

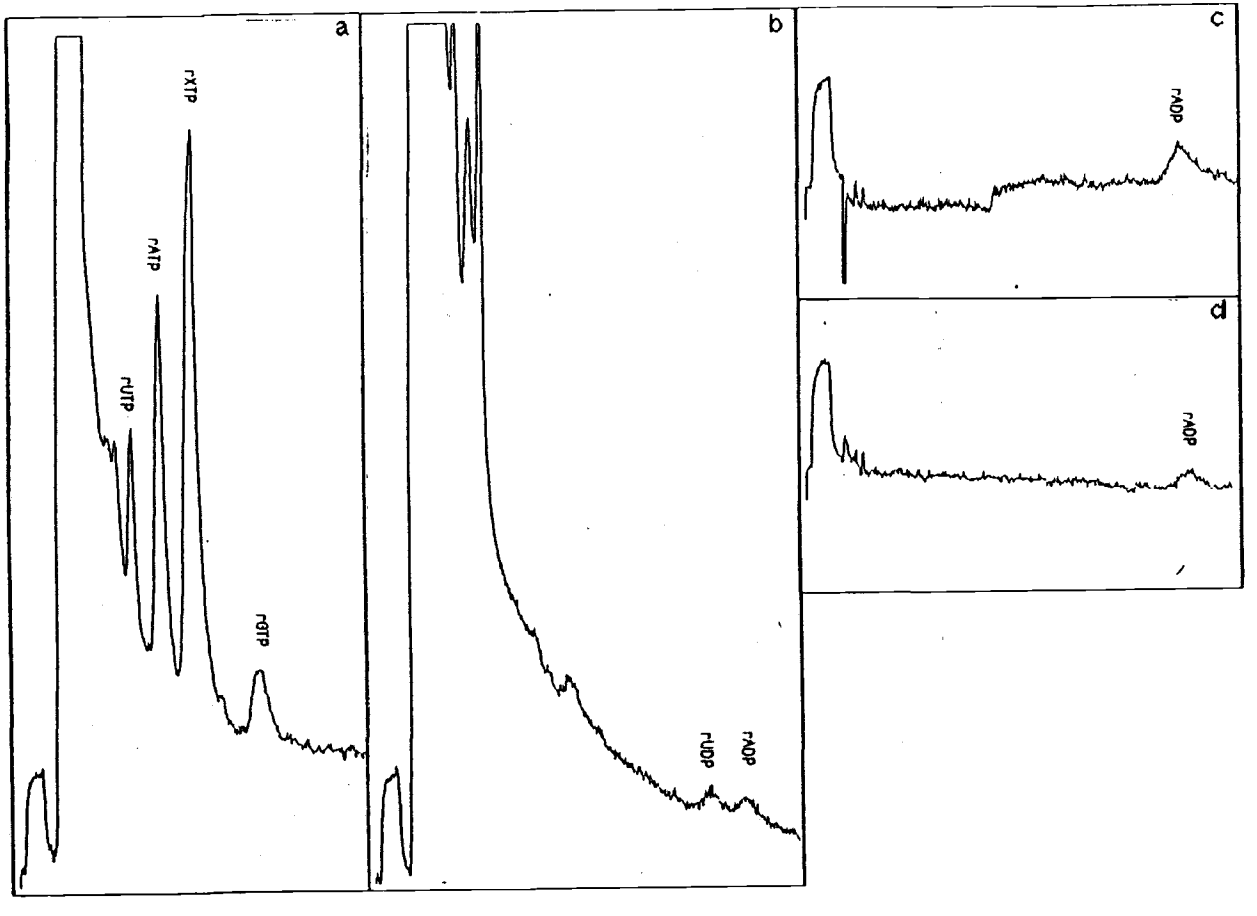


Figure 12. HPLC Profiles of Ribonucleoside Triphosphates and Ribonucleoside Diphosphates Recovered from Trichloroacetic Acid Extracts of Uninfected and T4 Wild Type Infected E. coli.

was not used in these studies because it usually requires large quantities of high-specific-activity [^{32}P]-orthophosphate and has limited sensitivity in measuring deoxyribonucleotide concentrations. Furthermore, as explained below, it is difficult to distinguish between dCTP and hmdCTP present in the same sample. The enzymatic dNTP assay, used in these studies, has typically been used for measuring dNTPs in extracts prepared from eucaryotic cells, but, to my knowledge, its use in measuring dNTPs from procaryotes has not been described. The enzymatic dNTP assay does not have the disadvantages of [32P]orthophosphate determinations, and by treatment of T4 extracts with purified dCTPase/dUTPase, hmdCTP and dCTP in the extracts could be measured.

Since this assay had not been used, before these studies, to measure dNTPs in extracts prepared from T4-infected cells, several control experiments were performed, to (1) optimize the assay conditions and (2) test for activities present in the extracts that might interfere with dNTP measurement. Two diagnostic controls can be done to show there are not any interfering activities introduced during cell treatment or nucleotide extraction. (1) Dilution of cell samples should be reflected by a linear decrease in dNTPs measured by the dNTP assay, and (2) the dNTP concentrations of samples, spiked with a known quantity of a given dNTP, should be additive.

These criteria are met for all four deoxyribonucleotides and both extraction protocols. Figure 13 shows typical results for just one deoxyribonucleotide, dATP. The polymerization reaction was completed by 75 minutes and maintained a stable plateau for at least 30 minutes thereafter. Measurement of dATP is linear with respect to dilution

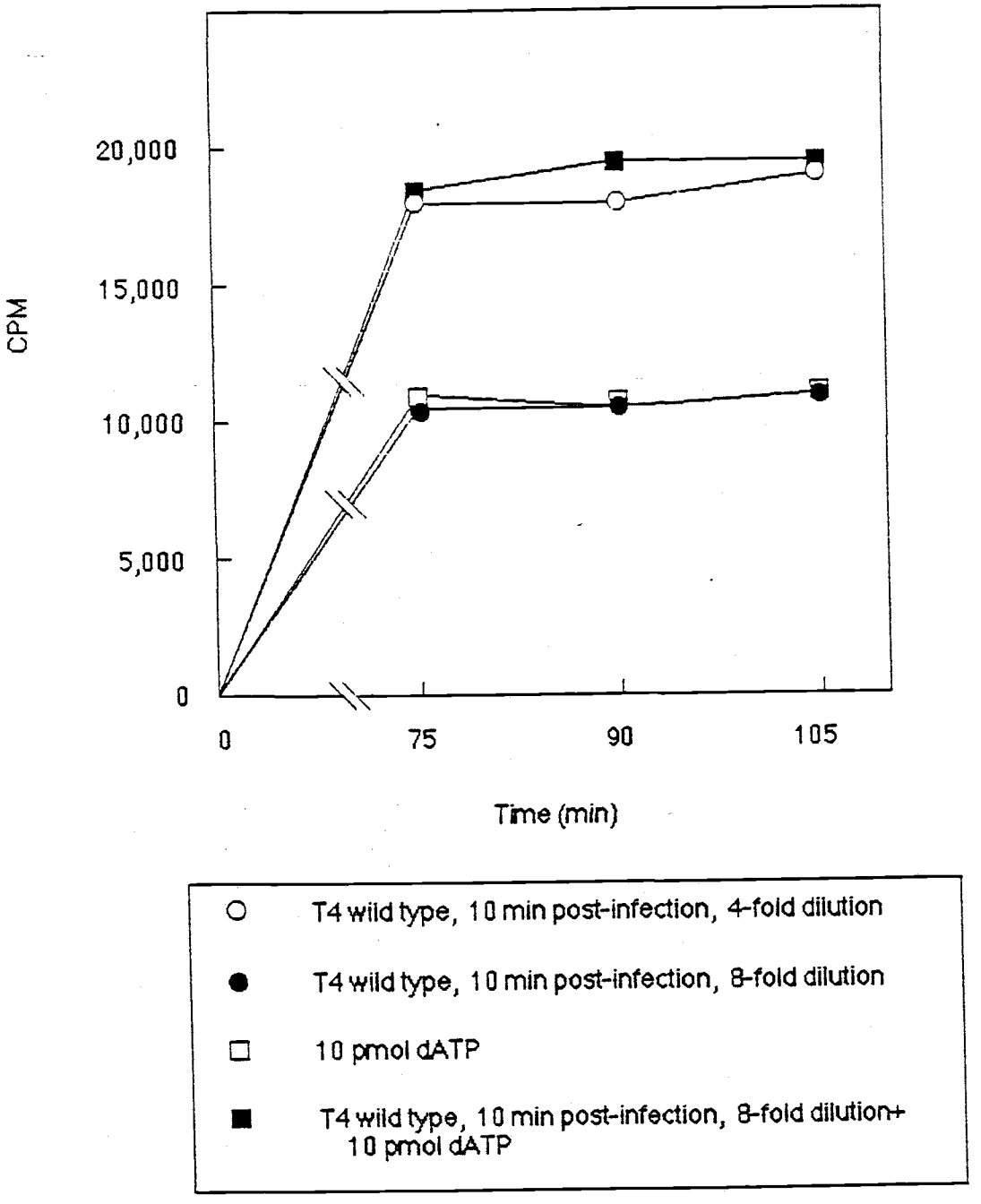


Figure 13. Time Course of the Enzymatic dNTP Assay for dATP in Trichloroacetic Acid Extracts. T4 wild type extracts were prepared as described in Fig. 11, and Materials and Methods.

(see also Fig. 14), and is additive when the cell sample is spiked with dATP. In extracts where there was greater than a 200-fold concentration in volume (volume of cell culture sampled/volume of final cell sample), undiluted cell samples frequently gave lower dNTP values than dNTPs measured in dilutions of the same extracts (Fig. 14). The most likely explanation for this observation is the presence of salts in the cell samples inhibiting the DNA polymerase in the dNTP assay. On dilution of these extracts, the salt concentrations should be reduced enough to relieve DNA polymerase inhibition.

B. Measurement of dCTP, hmdCTP, dTTP, and dUTP by the Enzymatic dNTP Assay

Normally, when using the enzymatic dNTP assay, it is not possible to distinguish between some naturally occurring deoxyribonucleotides, for example dCTP and hmdCTP or dUTP and dTTP, or deoxyribonucleotide analogs, e.g. BrdUTP and dTTP. Pretreatment of reaction mixtures with *E. coli* dUTPase has been used by Shlomai and Kornberg (1978) to destroy contaminating dUTP present in an *E. coli* cell-free replication system. T4 synthesizes a dCTPase/dUTPase that, once purified, can be utilized in the same way to allow measurement of dCTP and hmdCTP or dUTP and dTTP by the enzymatic dNTP assay.

Initial experiments indicated that a partially purified preparation of the enzyme could be used to degrade dCTP, and presumably dUTP, but leave dTTP and hmdCTP intact (Fig. 15). Since pretreatment of cell samples could be done *in situ* before measurement of treated extracts by the enzymatic assay, the same requirements used

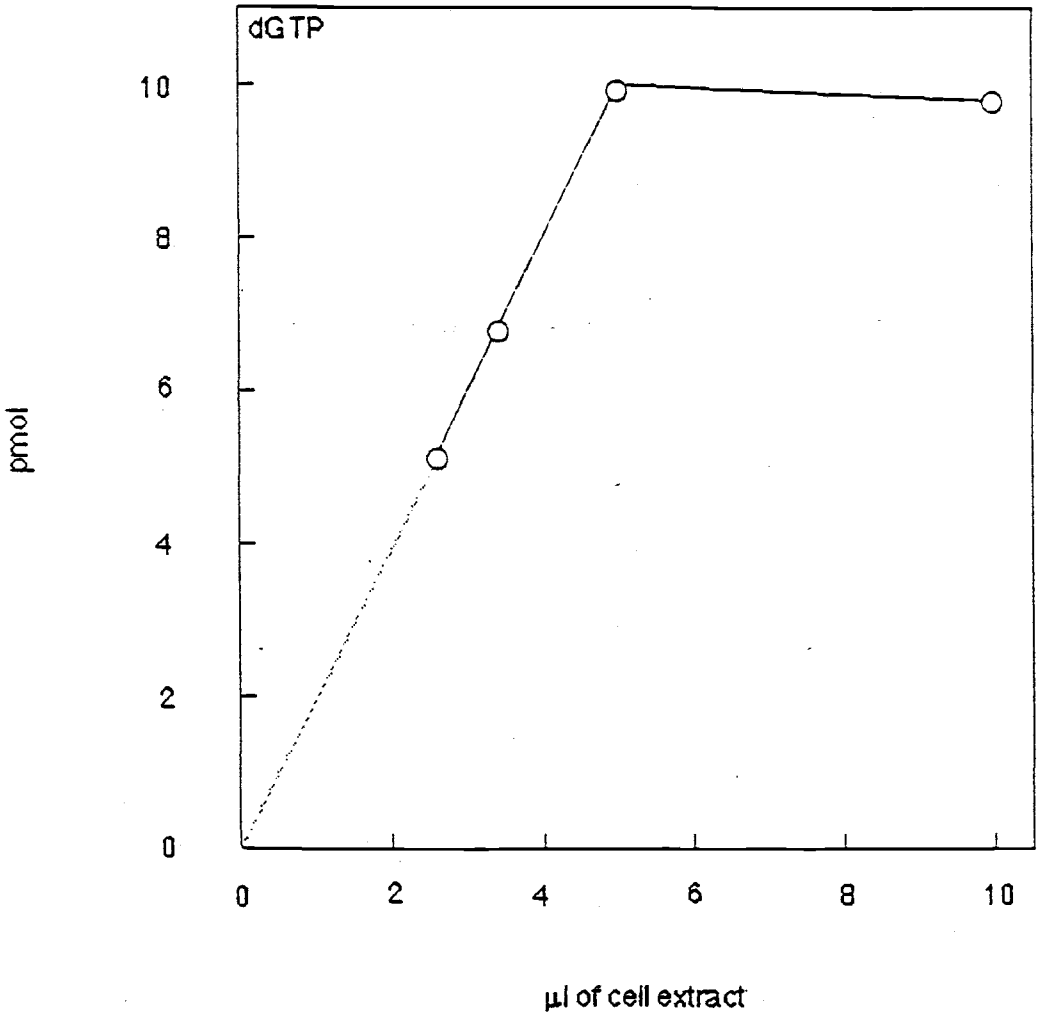


Figure 14. Inhibition of the dNTP Assay by T4 Extracts. TCA/TOA-prepared samples of T4-infected cells were concentrated 250-fold. The amount of cell sample in the dNTP assay is indicated on the X axis.

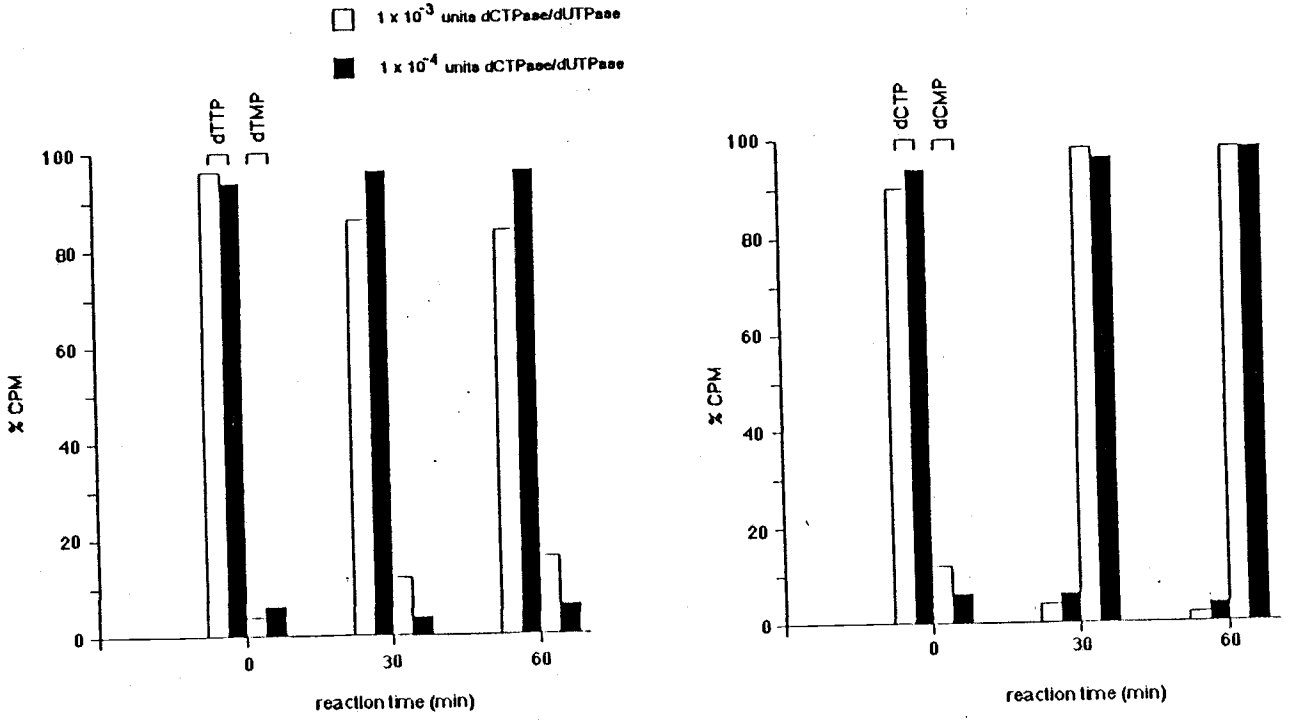


Figure 15. Treatment of dTTP and dCTP by dCTPase/dUTPase.

to judge the reliability of the dNTP assay with untreated cell samples should be met by dCTPase/dUTPase-treated samples. Treatment of known concentrations of dTTP, hmdCTP, dCTP, and dUTP (Fig. 16) or cell extracts (Fig. 17) with dCTPase/dUTPase gave identical results when compared to untreated samples. The exception, of course, was the degradation of dCTP by dCTPase/dUTPase in the 0 minute postinfection T4-infected samples. At this time during phage infections there should not be any hndCTP present in the phage-infected cells, since dCMP hydroxymethyltransferase has not yet been synthesized.

To summarize up to this point. (1) Nucleotide extraction by the TCA/TOA procedure is preferred over the MeOH/TCA/TOA procedure, because of the potential for nucleotide degradation during the latter. This degradation is presumed to be caused by activities coextracted during MeOH treatment that, if given the chance, can metabolize (deoxy)ribonucleotides before TCA treatment. (2) The dNTP assay performs well with extracts prepared by both extraction procedures. Samples that are concentrated 200-fold by volume during extract preparation must be diluted at least two-fold before measuring nucleotide concentrations with the dNTP assay. (3) Finally, in situ treatment of cell samples with dCTPase/dUTPase did not interfere with the subsequent measurement of dNTPs.

C. Deoxyribonucleotide Pool Imbalances and Altered Spontaneous Mutation Rates During Infections by T4 dCMP Deaminase Mutants

At least one dozen T4 phage gene products control aspects of dNTP biosynthesis in infected cells; therefore, mutations in most or all of

Figure 16. dNTP Assay of Deoxyribonucleoside Triphosphates in dCTPase/dUTPase-Treated dNTP Solutions. Reaction mixtures containing dNTP and 1 x 10^{-3} units T4 dCTPase/dUTPase (final volume of 20 µl) were incubated at 37°C for 30 minutes, at which time 80 µl of dNTP assay buffer (containing DNA polymerase, template, [^{3}H]dNTP complementary to the dNTP being measured, and buffer) was added. Acid precipitable counts were determined as described in Materials and Methods.

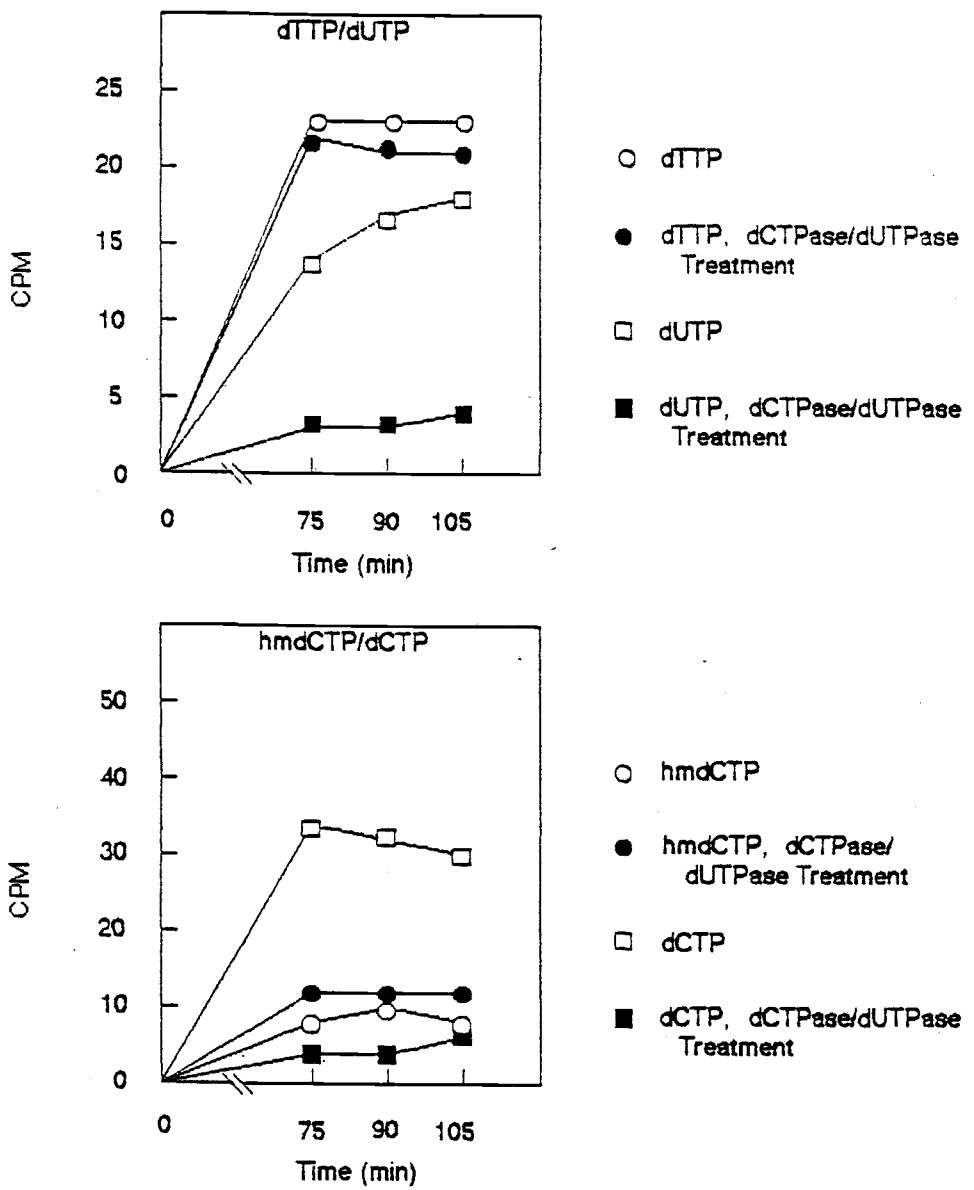


Figure 16. dNTP Assay of Deoxyribonucleoside Triphosphates in dCTPase/dUTPase-Treated dNTP Solutions.

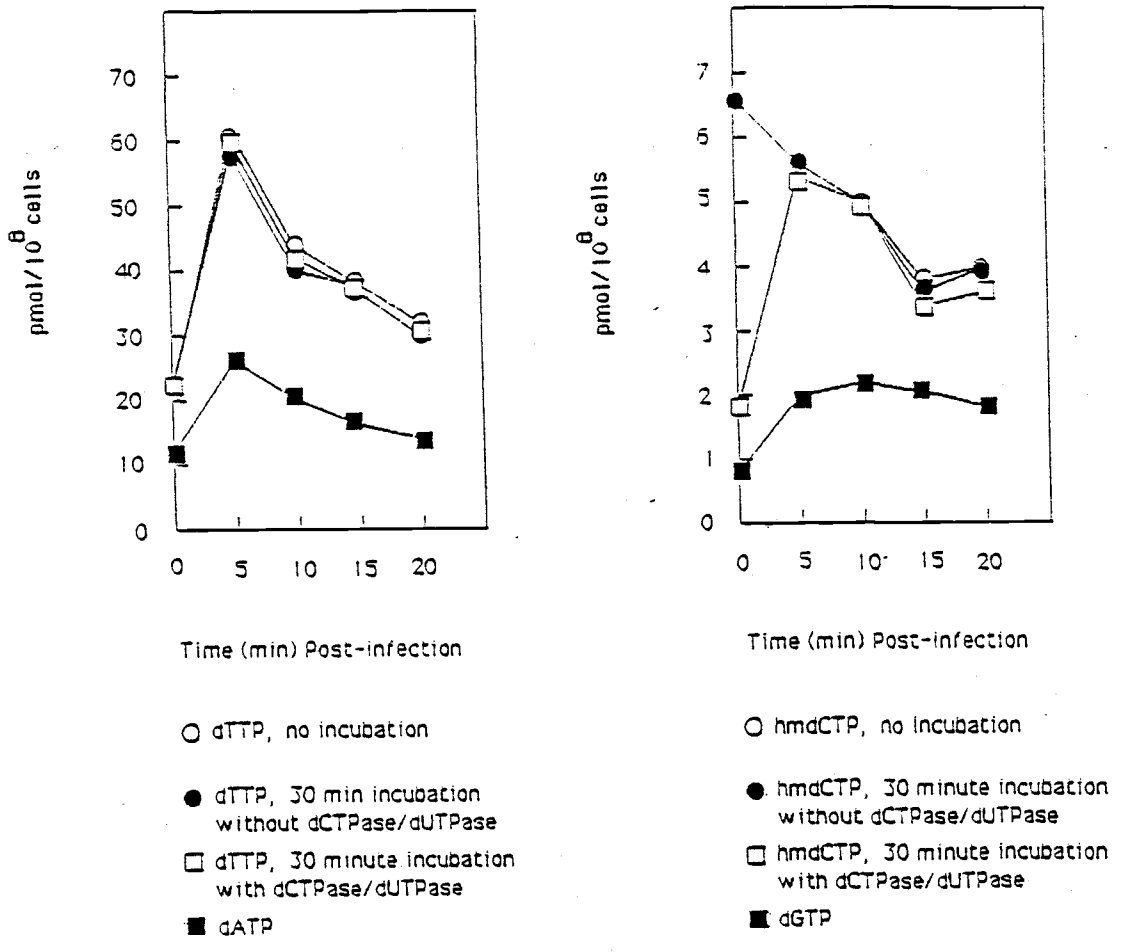


Figure 17. dNTP Pools Measured in dCTPase/dUTPase Treated T4 Extracts.

these genes can affect DNA precursor pools. In principle, mutations in any of these genes could be used for the studies described here; dNTP pool size changes would be correlated with the types and frequencies of mutations induced, as measured by reversion from a panel of well characterized rII mutations to wild type. My choice of the cd gene and its product, dCMP deaminase, for this initial study was based on the following considerations. (1) dCMP deaminase deficiencies in mammalian cells are mutagenic, but the specific mutations induced have not yet been identified; and (2) in T4 infections, as well as in mammalian cells, dCMP deaminase has been postulated to play a special role in controlling rates of pyrimidine deoxyribonucleotide biosynthesis.

I chose to use the T4 dCMP deaminase deletion mutant pseTΔ4 (Δcd, ΔpseT) for two reasons. First, the mutational studies described below required construction of a panel of doubly mutant phage containing well-defined mutations in the T4 rII genes, as markers to follow mutation rates, and a mutation in T4 dCMP deaminase. Normally, mutations in cd to not have a strong phenotype and cd mutant plaques are almost indistinguishable from wild type plaques when plated on E. coli. Thus, identification of double mutant recombinants is difficult. The pseTΔ4 deletion covers all or part of the dCMP deaminase structural gene and the closely linked gene which encodes polynucleotide kinase. Phage mutant in polynucleotide kinase do not plate on the host cell E. coli CTr5x (Snyder, 1983); hence, the pseT end of the deletion in pseTΔ4 confers an easily followed tag for the loss of dCMP deaminase. For most of my work, I used two "wild type" controls, namely, rII single mutants, which are mutant in neither dCMP

deaminase nor polynucleotide kinase, and the deletion mutant pseTΔ5. This deletion inactivates polynucleotide kinase but not dCMP deaminase, ensuring that observed abnormalities in dNTP pools or changed mutation frequencies resulted from the cd mutation, but not deletion of pseT.

Second, it is believed that during T4 infection a complex of physically associated enzyme activities involved with deoxyribonucleotide metabolism is formed (Mathews and Allen, 1983). This deoxyribonucleotide biosynthetic complex serves to regulate T4 dNTP biosynthesis and channel dNTPs into the T4 replication apparatus at actively growing replication forks, I felt, therefore, that total loss of an enzyme from the putative deoxyribonucleotide biosynthetic complex, rather than just loss of enzyme activity say through a missense mutation, would provide the most dramatic consequences in terms of disrupting either the biosynthetic complex or regulation of hmdCTP and dTTP pools by dCMP deaminase.

1. dNTP and rNTP Pools

As described above, the major pathway for dUMP biosynthesis in T4 infections and mammalian cells is catalyzed by dCMP deaminase, and loss of this enzyme in mammalian cells results in imbalanced dNTP pools. To determine the effect that loss of dCMP deaminase had on T4 deoxynucleotide metabolism and dNTP pools, nucleotide concentrations were measured at various times after phage infection (Fig. 18). The dNTP pools determined after infection by wild type phage, rIIamHB84, or pseTΔ5(cd^+, ΔpseT) were similar to results obtained with infection by pseTΔ5(cd^+, ΔpseT)/rIIamHB84 (data not shown). Furthermore, dNTP

Figure 18. Deoxyribonucleoside Triphosphate Pools Induced During psetΔ4(Δcd, ΔpseT)/rIIamHB84 and pseTΔ5(cd$^+$, ΔpseT)/rIIamHB84 Infections of E. coli B. Cells infected with pseTΔ4(Δcd, ΔpseT)/rIIamHB84 (0) or pseTΔ5(cd$^+$, ΔpseT)/rIIamHB84 (●) were harvested at the times indicated, and deoxyribonucleotides were measured as described in Materials and Methods. The values reported here are from a single experiment, but are comparable to dNTP concentrations determined by infections of E. coli B with the cd phage, pseTΔ4 or cdN16, or the cd$^+$ phage, pseTΔ5, rIIamHB84 or wild type T4. Note the expanded scale used for hmdCTP curves.

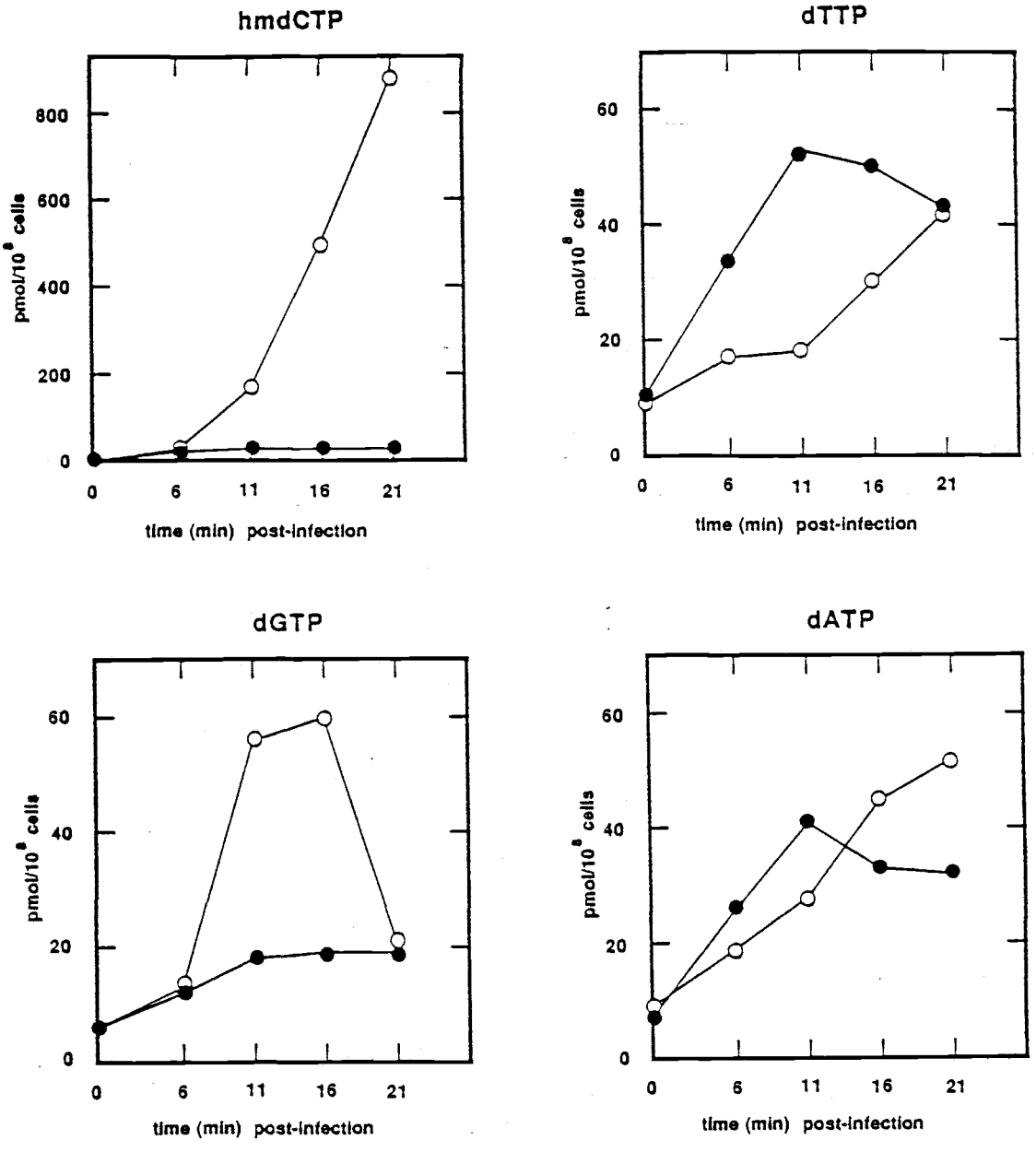


Figure 18. Deoxyribonucleoside Triphosphate Pools Induced During psetΔ4(Δcd, ΔpseT)/rIIamHB84 and pseTΔ5(cd$^+$, ΔpseT)/rIIamHB84 Infections of E. coli B.

pools measured after infection by pseTΔ4(Δcd, ΔpseT) or pseTΔ4 (Δcd, ΔpseT)/rIIamHB84 were the same as dNTP pools from infections by the T4 cd mutant cdN16 (data not shown). The most striking difference between pseTΔ5(cd^+, ΔpseT)/rIIamHB84 and pseTΔ4(Δcd, ΔpseT)/rIIamHB84 dNTP pools was the 30-fold expansion of the T4-induced hmdCTP pool in the pseTΔ4(Δcd, ΔpseT)/rIIamHB84 infection by about 20 minutes post-infection and the concomitant delay in accumulation of dTTP (Fig. 18). I also noted a transient 3- to 4-fold expansion of the dGTP pool during pseTΔ4(Δcd, ΔpseT)/rIIamHB84 infections, which paralleled the onset of hmdCTP accumulation. To summarize these results, loss of T4 dCMP deaminase caused a simultaneous accumulation of hmdCTP and deficiency of dTTP.

Pyrimidine ribonucleoside triphosphate pools changed somewhat during pseTΔ5(cd^+, ΔpseT)/rIIamHB84 infections, with the rUTP pool expanding 3-fold and the rGTP pool 5-fold relative to uninfected cells (Fig. 19). Loss of dCMP deaminase in the pseTΔ4(Δcd, ΔpseT)/rIIamHB84 infections, however, resulted in an 8- to 10-fold expansion of both pyrimidine rNTP pools and, in contrast to pseTΔ5(cd^+, ΔpseT)/rIIamHB84 infection, the rGTP pool did not significantly change.

2. Reversion Tests

Spontaneous mutation rates and the mutagenic pathways stimulated during pseTΔ4(Δcd, ΔpseT) or pseTΔ5(cd^+, ΔpseT) infections were measured by following reversion at well defined loci in the T4 rII genes. To confirm that any changes I observed in mutation rates were due to the deficiency in dCMP deaminase and not from deletion of pseT,

Figure 19. Changes in Ribonucleoside Triphosphate Pools Induced During pseTΔ4(Δcd, ΔpseT)/rIIamHB84 and pseTΔ5(cd$^+$, ΔpseT)/rIIamHB84 Infections of E. coli B. Extracts of phage-infected E. coli B used to measure dNTP pools were analyzed by HPLC to determine rNTP levels. The abnormally large hndCTP accumulation, late during cd infections, made it necessary to correct for the contribution of hmdCTP to the HPLC-determined rCTP values. The relative rNTP values reported here represent the ratio of rNTP present at the indicated times post-infection, to rNTP values determined at 0 minutes post-infection. The median rNTP concentrations of three 0-minute post-infection extracts, which represent rNTP concentration of uninfected E. coli, were: rUTP, 0.82 mM; rCTP, 0.83 mM; rATP, 1.6 mM; and rGTP, 0.6 mM. These values are comparable to those measured by Mathews (1972).

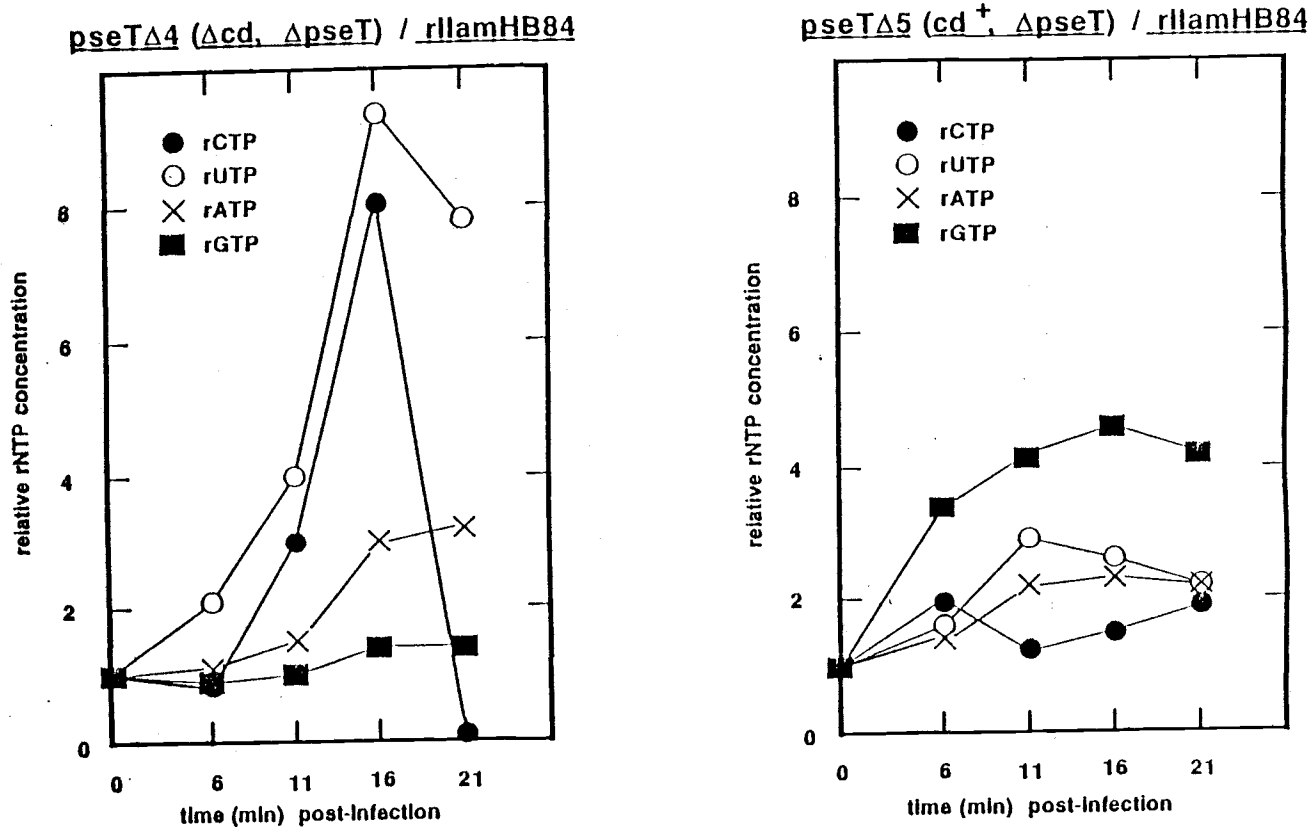


Figure 19. Changes in Ribonucleoside Triphosphate Pools Induced During pseTΔ4(Δcd, ΔpseT)/rIIamHB84 and pseTΔ5(cd+, ΔpseT)/rIIamHB84 Infections of E. coli B.

mutation rates were determined for the respective rII single mutants, in parallel with the pseTΔ4(Δcd, ΔpseT)/rII or pseTΔ5(cd^+, ΔpseT)/rII double mutants. And, as shown in Table V, deletion of polynucleotide kinase alone, in the pseTΔ5(cd^+, ΔpseT)/rII reversion tests, had no effect on the mutation rates of any rII mutants used in this study.

E. coli B were used as host cells throughout this investigation. Stocks of rII phage grown on these cells will undergo selection for rII^+ revertants (Drake, 1970); hence, the spontaneous mutation rates reported here are slightly higher than those values determined by other laboratories. Comparison of mutation rates for rIIamHB74 or rIIUV215, measured on E. coli, and the nonselective host for rII^+ reversion, E. coli BB, are shown in Table V. As expected, the absolute mutation rates were lower for rIIamHB74 and pseTΔ4(Δcd, ΔpseT)/rIIamHB74 when grown on E. coli BB. The relative increase in reversion of pseTΔ4/rIIamHB74 over rIIamHB74 when grown on E. coli BB cells, however, is still significant (sixfold) compared to the tenfold increase when grown on E. coli B. Since stimulation of reversion by pseTΔ4 is at least five- to tenfold for all the AT-to-GC reverters when grown on E. coli B, I believe these data reflect a genuine consequence of the nucleotide pool imbalance, and are not an artifact of using E. coli B for the reversion tests.

Most of the rII mutants selected for this study were chosen because they mapped onto a region of the rIIB gene previously sequenced (Pribnow et al., 1981). Hence rII^+ revertants of those mutant alleles could be sequenced and compared to the parental wild type sequence. I also used the rIIUV215 and rIIUV363 mutants, however, for measuring reversion rates, because data from other

Table V. Stimulation of Mutation Rates by Loss of T4 dCMP Deaminase. The rII mutants used in this study, and their reversion pathways, have been described by others (Smith et al., 1973; Williams and Drake, 1977). Mutation rates were measured as described in Materials and Methods, and the values reported here are the median of parallel reversion tests using three independent isolates of each phage. Three-fold or greater changes in mutation rates are considered to be significant (Drake, 1970).

Table V. Stimulation of Mutation Rates by Loss of T4 dCMP Deaminase.

PHAGE	*rII* REVERSION PATHWAY	*rII*$^+$ REVERTANTS PER 10^8 PHAGE	
		B cells	BB cells
rIIamHB84	AT to GC	1	
pseTΔ5(cd$^+$)/rIIamHB84	and	1	
pseTΔ4(Δcd)/rIIamHB84	transversions	10	
rIIamHB74	AT to GC	9	1
pseTΔ5(cd$^+$)/rIIamHB74	and	15	-
pseTΔ4(Δcd)/rIIamHB74	transversions	120	6
rIIUV215	AT to GC	33	14
pseTΔ5(cd$^+$)/rIIUV215		39	21
pseTΔ4(Δcd) /rIIUV215		52,000	92,000
rIIHD263	AT to GC	4	
pseTΔ5(cd$^+$)/rIIHD263		4	
pseTΔ4(Δcd)/rIIHD263		22	
rIIUV363	GC to AT	18	
pseTΔ5(cd$^+$)/rIIUV363		50	
pseTΔ4(Δcd)/rIIUV363		25	
rIISN103	GC to AT	19	
pseTΔ5(cd$^+$)/rIISN103		21	
pseTΔ4(Δcd)/rIISN103		6	
rII514	Frameshift	200	
pseTΔ5(cd$^+$)/rII514		140	
pseTΔ4(Δcd)/rII514		160	
rIIFC11	Frameshift	150	
pseTΔ5(cd$^+$)VrIIFC11		81	
pseTΔ4(ΔcdVrIIFC11		250	

laboratories indicated that these alleles have extraordinarily high reversion rates under conditions that should induce certain dNTP pool imbalances (Smith et al., 1973; Williams and Drake, 1977). Indeed, reversion measured at the rIIUV215 site was stimulated about 100-fold in both the pseTΔ4(Δcd, ΔpseT)/rIIUV215 (Table V) and cdN16/rIIUV215 double mutants (Table VII). In comparison, the other three AT-to-GC reverting pseTΔ4/rII double mutants had only a five-to tenfold stimulation in reversion rates.

Reversion of the rII frameshift mutants tested here was not significantly affected by pseTΔ4. However, there was an apparent decrease in mutation rates for the GC-to-AT-reverting pseTΔ4/rIISN103. While this result is somewhat inconclusive, as only one GC-to-AT reverter was affected and the mutation rate decrease was barely significant (about threefold), it is the response expected if the pseTΔ4 dNTP pool imbalance was antimutagenic by decreasing the formation of G:T mispairs. In fact, the pseTΔ4 hmdCTP/dTTP imbalance should ensure the formation of correct G:C basepairs during replication. The decreased pseTΔ4/rIISN103 mutation rate is interesting, as antimutagenic effects originating from mutations in T4 deoxyribonucleotide metabolism have been described only for temperature-sensitive gene 42 (dCMP hydroxymethyltransferase) mutants of T4 (Williams and Drake, 1977). In that study, however, it was not established whether antimutagenesis was a result of dNTP pool imbalances or altered protein-protein interactions. Moreover, the antimutagenic effect was not seen in all sites examined. The pseTΔ4 antimutagenic effect will be better established in a more extensive study, using several different GC-to-AT-reverting pseTΔ4/rII double

mutants and conditions amplifying the reduction in reversion rates.

3. Sequencing

Since amber terminator codons can revert to non-terminating codons by AT-to-GC transitions at either the first or second position of the amber codon or by transversions at any position in the amber codon, the nucleotide substitution giving amber revertants cannot always be unambiguously determined by rII amber-revertant plating phenotypes. I recovered and sequenced several rIIamHB74$^+$ revertants to determine the base substitution and the substitution position within the revertant amber codon.

The rIIamHB74 mutation originated via a GC-to-AT transition at nucleotide 528 (Pribnow et al., 1981) in the rIIB cistron of T4, which maps into a region of rIIB known to be dispensable for the function of rIIB gene product (Crick et al., 1961; Barnett et al., 1967). Since this domain of the rIIB polypeptide is dispensable, any amino acid ultimately encoded by reversion of the amHB74 codon should be tolerated by the rIIB protein, yielding a functionally wild type protein and preventing selection for or against any particular base substitution. AT-to-GC transitions at nucleotides 528 or 527 in rIIamHB74 will generate nonterminator codons, with the original wild type (TGG) tryptophan codon regenerated by an AT-to-GC transition at nucleotide 528 and a pseudo-wild type (CAG) glutamine codon by AT-to-GC transitions at nucleotide 527.

Figure 20 is an autoradiogram of a sequencing gel showing the nucleic acid sequence of the region surrounding the rIIamHB74 mutation

Figure 20. Autoradiogram of an 8% Acrylamide/7 M Urea Sequencing Gel Showing the Nucleic Acid Sequence of the rIIamHB74 Mutation and rII^{+} Revertants of rIIamHB74, pseTΔ4/rIIamHB74, and pseTΔ5/rIIamHB74.

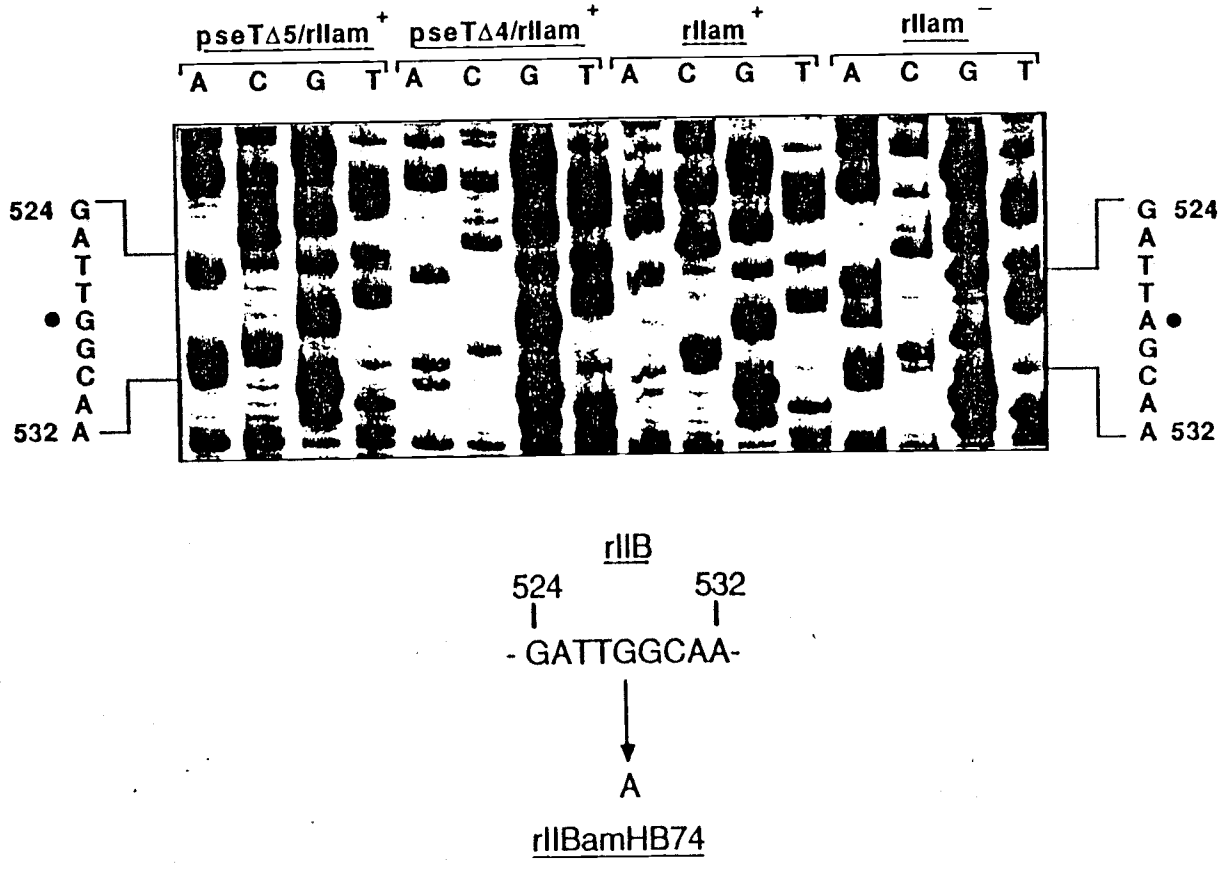


Figure 20. Autoradiogram of an 8% Acrylamide/7 M Urea Sequencing Gel Showing the Nucleic Acid Sequence of the rIIamHB74 Mutation and rII+Revertants of rIIamHB74, pseTΔ4/rIIamHB74, and pseTΔ5/rIIamHB74.

and rIIamHB74$^+$ revertants or rIIamHB74, pseTΔ5(cd$^+$, ΔpseT)/rIIamHB74 and pseTΔ4(Δcd, ΔpseT)/rIIamHB74. The revertant sequences shown in this gel all have AT-to-GC transitions at position 528 regenerating the wild type (TGG) tryptophan codon. Table VI summarizes the results from sequencing a limited number of the rIIamHB74$^+$ revertants. The most frequently recovered revertants had AT-to-GC transitions at the second position of the (TAG) amber codon with one exception, a pseTΔ5(cd$^+$, ΔpseT)/rIIamHB74$^+$ revertant, by an AT-to-GC transition at the first position in the amber codon (Table VI).

D. Thymidine Reversal of T4 dCMP Deaminase Mutant-Induced dNTP Pool Imbalances and the Effects on Mutation Rates

If the pseTΔ4 dNTP pool imbalance is the sole cause of the increased AT-to-GC mutation rates, then returning the pseTΔ4 dNTP concentrations to wild type levels should concurrently reduce AT-to-GC mutation rates, possibly to wild type levels. Mammalian cells that have a similar dCTP/dTTP pool imbalance from a mutation in rCTP synthetase, respond in this fashion to the addition of thymidine. Spontaneous mutation rates are seen to decrease with increasing thymidine concentration in the growth medium, and concurrent normalization of intracellular dNTP pools (Meuth, 1981; Weinberg et al., 1985).

The effect of thymidine on pseTΔ4 and pseTΔ5 dNTP pools is shown in Figure 21. The pools were measured at 10 minutes post-infection in the presence of increasing thymidine. As expected, the dTTP pools increased for both pseTΔ4 and pseTΔ5 infections with increasing

Table VI. Nucleic Acid Sequences of *rII*$^{+}$ Revertants Isolated from *rIIamHB74*, *pseTΔ4/rIIamHB74*, and *pseTΔ5/rIIamHB74*$^{+}$ Infections.

	REVERTANTS	
	TAG ↓	TAG ↓
MUTANT	TGG (Trp)	CAG (Gln)
rIIamHB74	3	-
pseTΔ5/rIIamHB74	-	1
pseTΔ4/rIIamHB74	6	-

Figure 21. Effects of Thymidine on pseTΔ4(Δcd, ΔpseT) and pseTΔ5(cd^+, ΔpseT) dNTP Pools. E. coli B cells were grown to 2 to 5 x 10^8 cells/ml, at 37°C in SM9 without TdR. Immediately before infection, thymidine was added to the indicated concentration, and phage were added to a concentration of about 5 phage/cell. Phage-infected cultures were harvested at 10 minutes post-infection and nucleotides extracted by the MeOH/TCA/TOA procedure.

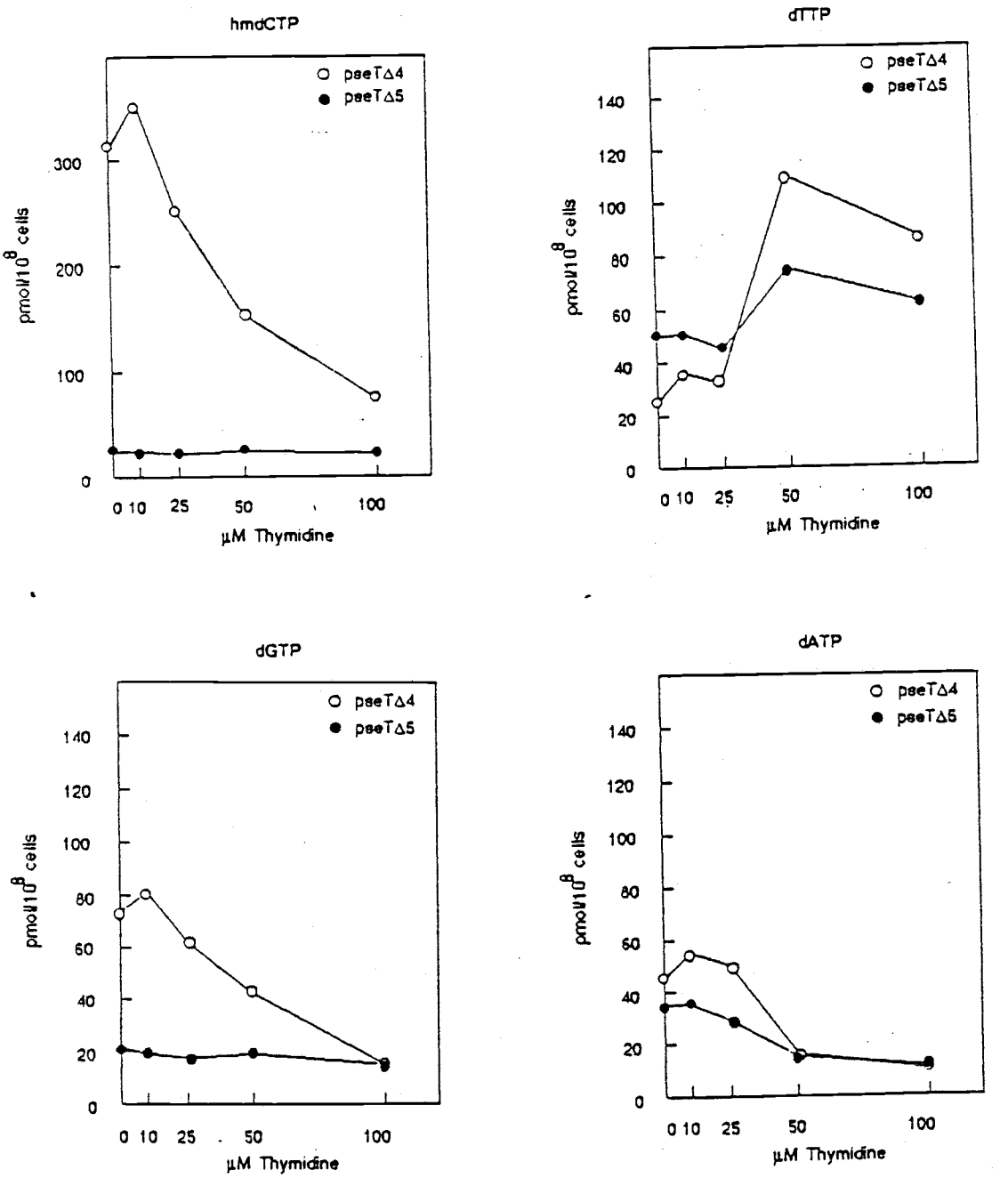


Figure 21. Effects of Thymidine on pseTΔ4(Δcd, ΔpseT) and pseTΔ5(cd+, ΔpseT) dNTP Pools.

thymidine, whereas during pseTΔ4 infections dATP, dGTP and hmdCTP were all reduced to almost wild type levels by 50 to 100 μM thymidine. However, pseTΔ5 dNTP pools were changed much less, with the only appreciable changes seen in the decreased dATP and enlarged dTTP pools.

Intracellular rNTP pools, for both pseTΔ4 and pseTΔ5 infections, were insignificantly changed by addition of thymidine (Fig. 22).

The mutation rates of the cd^+ wild type controls, rIIUV215 and pseTΔ5/rIIUV215, were not changed in the presence of 200 μM thymidine (Table VII). When one considers the response of the pseTΔ4 dNTP pools to thymidine, it was surprising that there was only a 2.6-fold decrease in pseTΔ4(Δcd, ΔpseT)/rIIUV215 reversion in the presence of 200 μM thymidine, and no change for the dCMP deaminase missense mutant, cdN16(cd, $pseT^+$)/rIIUV215. While the decrease in pseTΔ4(Δcd, ΔpseT)/rIIUV215 mutation rates at 200 μM thymidine are not large enough to be considered a significant response, reduced AT-to-GC reversion is the expected change on normalization of the dNTP pools to wild type levels. The implications of pseTΔ4-stimulated mutagenesis and response to thymidine versus that of cdN16 are considered in the Discussion.

E. Spontaneous and BrUdR-Induced Mutagenesis of T4 Ribonucleotide Reductase Mutants

It should be possible to alter deoxyribonucleotide metabolism by substitution of native metabolic enzymes with comparable enzymes from different organisms, which are distinguished by having different

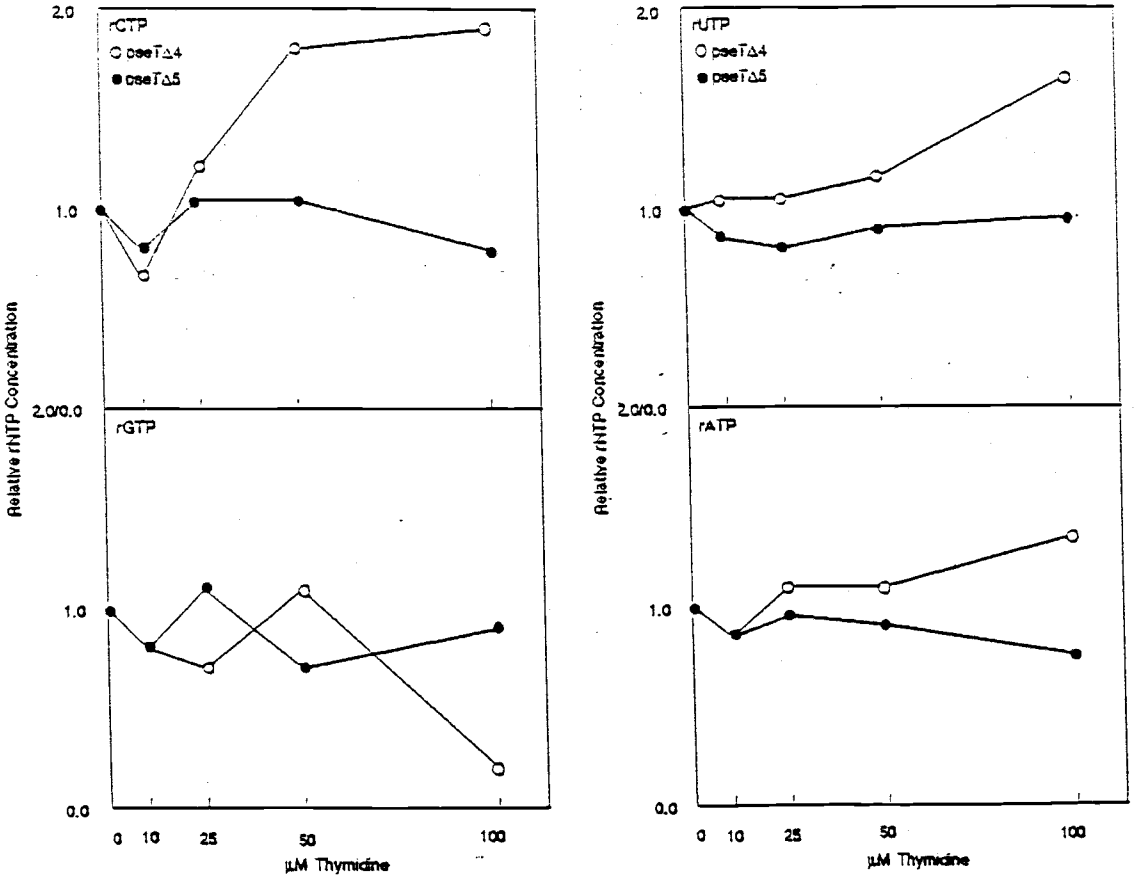


Figure 22. Effects of Thymidine on pseTΔ4(Δcd, ΔpseT) and pseTΔ5(cd$^+$, ΔpseT) rNTP Pools. The samples prepared for dNTP measurements (Fig. 21) were used for measurement of rNTPs by HPLC.

Table VII. Effects of Thymidine on the Mutation Rates of rIIUV215, pseTΔ5/rIIUV215, pseTΔ4/rIIUV215, and cdN16/rIIUV215.

	rII⁺REVERTANTS x 10^7	
PHAGE	-TdR	+TdR(200uM)
rIIUV215	1 (n=6)	1 (n=6)
pseTΔ5/rIIUV215	2 (n=6)	3 (n=6)
pseTΔ4/rIIUV215	9,200 (n=3)	3500 (n=6)
cdN16/rIIUV215	11,400 (n=2)	10,000 (n=5)

( ) = number of samples used to determine medians.

Reversion rates were measured in minimal medium (SM9), using E. coli BB cells, with or without thymidine. Phage are described in the text, but their genotypes are summarized here: rIIUV215(cd⁺, pseT⁺, rII⁻) reverts by AT-to-GC transitions, pseTΔ5/rIIUV215(cd⁺, ΔpseT, rII⁻), pseTΔ4/rIIUV215(Δcd, ΔpseT, rII⁻), cdN16/rIIUV215(cd⁻, pseT⁺, rII⁻).

regulatory properties. This may have several consequences, including: (1) altered deoxyribonucleotide metabolism such that a new balance of dNTPs is synthesized, or (2) change the organism's metabolic response to antimetabolites and nucleotide analogs. In turn, if, under appropriate conditions, the substituted enzyme establishes a new balance of dNTPs synthesized, then replication fidelity should be affected.

One particularly interesting target for enzyme substitution is ribonucleotide reductase. Since ribonucleotide reductase sits at the head of deoxyribonucleotide metabolism, it can potentially establish the relative concentrations of deoxyribonucleoside triphosphates simply by catalyzing reduction of ribonucleoside diphosphates in different proportions. Ribonucleotide reductase purified from T4-infected E. coli and uninfected E. coli are distinctly different proteins and have distinctly different patterns of regulation governing the enzymes' affinity for each of the ribonucleoside diphosphate substrates (Thelander and Reichard, 1979). Hence, at the beginning of this study, I anticipated that T4-intracellular deoxyribonucleoside triphosphate pools would be altered when E. coli ribonucleotide reductase was substituted into T4 deoxyribonucleotide metabolism.

It was first necessary to construct a host cell line overexpressing E. coli ribonucleotide reductase for the following reasons. On infection of E. coli, T4 induces at least a 10- to 50-fold increase of virus ribonucleotide reductase activity over that already present in the cell (Yeh et al., 1969). Uninfected cell deoxyribonucleotide metabolism is probably incapable of supplying the

volume of deoxyribonucleoside triphosphates needed for T4 DNA synthesis. And, it is predicted that reduced rates of DNA synthesis from limiting dNTP concentrations will, in itself, result in altered mutation rates (Galas and Branscomb, 1978; Clayton et al., 1979). The two cell lines eventually created were: ED8689/pPS2, an E. coli line containing a plasmid encoding both subunits of the E. coli ribonucleotide reductase, and known to overexpress the enzyme in E. coli HB101 (Platz and Sjöberg, 1980); and ED8689/pBR322, a cell carrying a related plasmid that does not encode ribonucleotide reductase, and should have normal cell levels of E. coli ribonucleotide reductase. Infections of the E. coli ribonucleotide reductase-overexpressing cell line, ED8689/pPS2, by doubly mutant virus containing both the nrdBamB55 mutation and one of the well-characterized rII mutants described above, were used to determine T4 mutation rates when E. coli ribonucleotide reductase substitutes for T4 ribonucleotide reductase.

T4 virus production is limited to about 163 phage per cell during infections of non-overexpressing ED8689/pBR322 by T4 nrdBamB55. This is in contrast to infections by wild type T4 of the same cell line which yield almost 700 phage per cell (Table VIII). Virus production in infections of the overexpressing E. coli ribonucleotide reductase cell line ED8689/pPS2 by the T4 nrdBamB55 mutant (about 1000 virus per cell), however, exceeds that of wild type T4 production and indicates that E. coli ribonucleotide reductase can functionally substitute for the T4 enzyme.

Does substitution of E. coli reductase for the T4 enzyme increase mutation rates? The data in Table IX are somewhat inconclusive in

Table VIII. Phage Burst Sizes of nrdBamB55-infected ED8689/pBR322 and ED8689/pPS2.

	PHAGE PER CELL	
	CELLS	
	ED8689/pBR322	ED8689/pPS2
Wild Type	1 (684)	0.8 (511)
nrdBamB55	0.2 (163)	1.6 (1093)

() = phage produced per cell, other values are relative to wild type infections of ED8689/pBR322.

Phage burst sizes were measured at 90 minutes post-infection (37°C). ED8689/pPS2 are cells carrying the pPS2 plasmid, encoding both subunits of E. coli ribonucleotide reductase, and overproduce the enzyme. ED8689/pBR322 are cells containing the pBR322 plasmid and have normal levels of ribonucleotide reductase.

Table IX. BrUdR-Stimulated Mutagenesis of rIIUV215, rIISN103, nrdBamB55/rIIUV215, and nrdBamB55/rIISN103.

		Relative Reversion Rates			
		BrUdR Concentration			
Phage	Cells	0 uM	30 uM	100 uM	300 uM
rIIUV215	ED8689/pPS2	1(5.4)	264	200	4
nrdBamB55/rIIUV215	ED8689/pPS2	1(5.2)	740	1346	330
rIISN103	ED8689/pPS2	1(0.2)	9359	10,227	18,450
nrdBamB55/rIISN103	ED8689/pPS2	1(0.5)	763	3646	5706
rIIUV215	ED8689/pBR322	1(1.4)	357	702	179
rIISN103	ED8689/pBR322	1(0.1)	19,441	31,667	18,933

Values in parentheses are absolute reversion rates x 10^7. The remaining data have been normalized to the 0 µM BrUdR reversion rates. rIIUV215 reverts by AT-to-GC transitions, and rIISN103 by GC-to-AT transitions.

Cells used for reversion tests were grown in the presence of tetracycline (12.5 µg/ml). Reversion tests were performed in minimal medium (SM9) containing the indicated amounts of BrUdR, without tetracycline. All BrUdR experiments were carried out in the dark or under a red light.

answering this question. There is about a 4-fold increase in the mutation rates of both nrdBamB55/rII mutant infections, when comparing the mutation rates of nrdBamB55/rII on ED8689/pPS2 with the mutation rates of nrd^+/rII on ED8689/pBR322. This a weak response (a 3-fold change in mutation rates is the minimum change needed to be considered a mutagenic response), and both AT-to-GC and GC-to-AT mutation rates were increased. One characteristic of deoxyribonucleotide pool imbalance-stimulated mutation rates is that one base substitution pathway is usually more strongly stimulated than the other, and reflects the nature of the imbalance. One example is the increased AT-to-GC mutation rates observed as a result of decreased dTTP pools and expanded dCTP pools during cd infections. The results in Table IX suggest that possibly some mechanism other than simple mass action-directed deoxyribonucleotide misincorporation is responsible for the mutation rates in nrdBamB55 infections.

Since E. coli and T4 ribonucleotide reductases are regulated differently by their effectors (Thelander and Reichard, 1979), I was interested in identifying the mutagenic response of T4 nrdBamB55/rII virus to the deoxyribonucleoside analog, bromodeoxyuridine (BrUdR). This analog is metabolized to the deoxyribonucleoside triphosphate, BrdUTP, when added to the medium of growing E. coli. The mutagenic effects of BrdUTP have been studied in both T4-infected and uninfected E. coli, and it is known to stimulate predominantly AT-to-GC transitions in E. coli (Howard and Tessman, 1964; Skopek and Hutchinson, 1982) and GC-to-AT transitions in T4 (Champe and Benzer, 1962). It has been suggested by other laboratories that the opposite mutagenic pathways stimulated by BrdUTP in T4-infected and uninfected

E. coli are determined in part by the relative BrdUTP concentration in the intracellular deoxyribonucleoside triphosphate pools (Hopkins and Goodman, 1980). If E. coli or T4 ribonucleotide reductase is the enzyme responsible for establishing deoxyribonucleoside triphosphate pools and consequently determining the mutagenic response to BrUdR, exchanging the ribonucleotide reductases should also change the mutagenic response of T4 or E. coli to BrUdR. In fact, in the presence of BrUdR, At-to-GC mutations are increased 6- to 8-fold during nrdBamB55/rIIUV215 infections of ED8689/pPS2, while GC-to-AT mutations are reduced (Fig. 23, Table IX). It was somewhat surprising that by 30 μM BrUdR, mutagenesis was independent of BrUdR concentration (Table IX, Fig. 23). This suggests that BrdUTP, at relatively low concentrations, has effectively saturated the respective deoxyribonucleotide pool with which it is competing for incorporation into DNA.

F. Deoxythymidine Triphosphate Pools in Cells Overexpressing Thymidylate Synthase

As mentioned in the Introduction, the relatively low Vmax measured for thymidylate synthase and dCMP hydroxymethyltransferase make these enzymes potential bottlenecks in deoxyribonucleotide metabolism. Likewise, if the intracellular enzyme concentration is limiting, thymidylate synthesis will be incapable of pacing the demand for dTTP. With these considerations in mind, overexpression of thymidylate synthase should be expected to expand intracellular dTTP pools, or rescue cell and phage growth under conditions when thymidylate demand

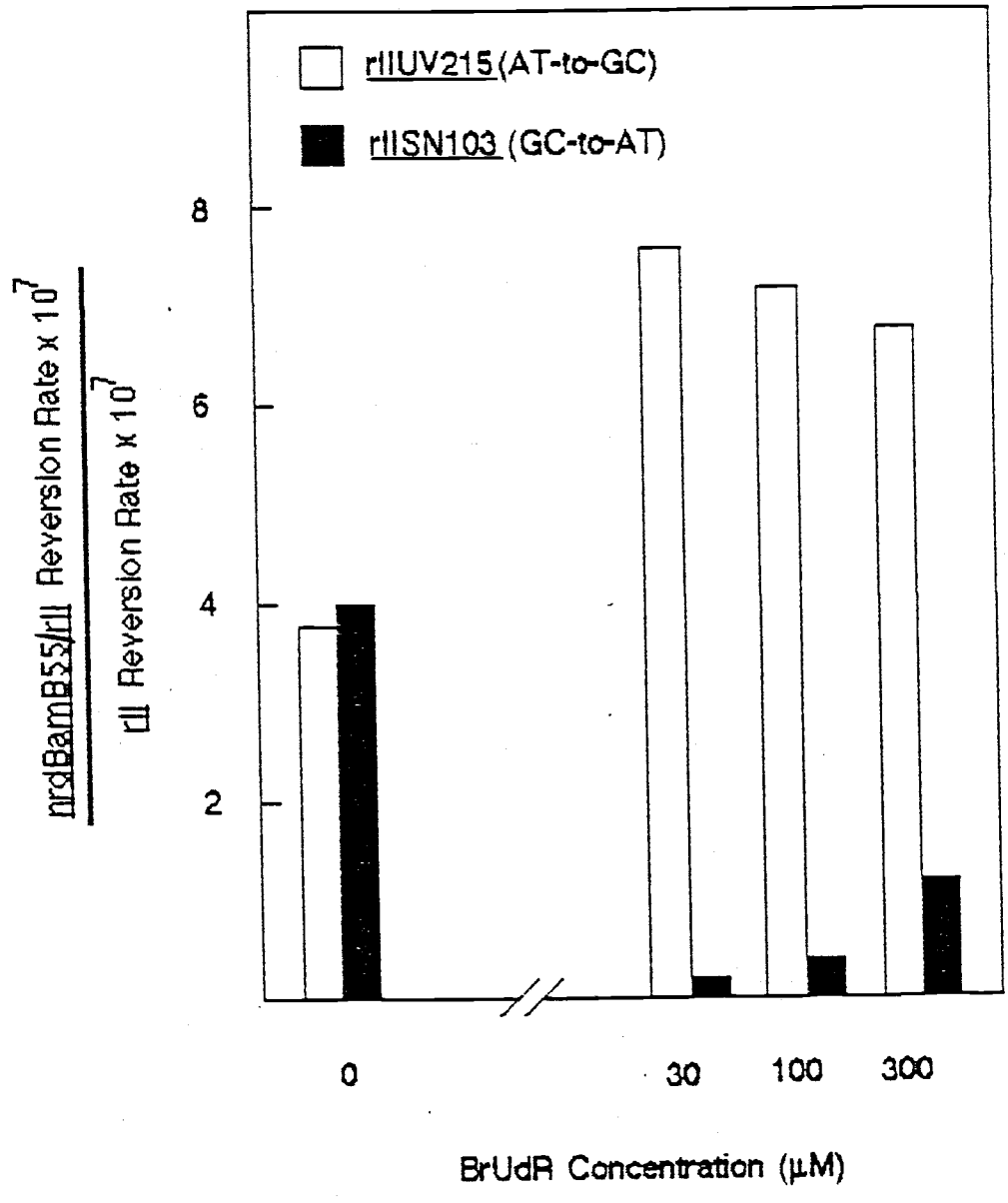


Figure 23. Changes in BrUdR-Stimulated Mutagenesis with Loss of T4 Ribonucleotide Reductase. The nrdBamB55/rII mutation rates were those determined on ED8689/pPS2, and rII-single mutation rates were determined on ED8689/pBR322 (Table IX).

exceeds the capacity of wild type enzyme activity to supply dTMP.

Two E. coli cell lines carrying plasmids constitutively overexpressing a Bacillus subtilis-related thymidylate synthase were tested for the effects of increased thymidylate synthase activity on cellular dTTP pools. The enzyme levels for MB61.21 and MB61.22 were substantially higher when compared to enzyme levels of parental cells (Table X), but were 3-fold less than those determined by Belfort (personal communication). Cellular dTTP pools, however, were relatively unchanged in both thymidylate synthase overexpressors (Table XI).

Since dTTP pools were seen earlier to expand during T4 infections of E. coli B cells (Fig. 17 and 18), I decided to measure these pools during infections with doubly mutant phage, td8nd28, which synthesizes an inactive T4 thymidylate synthase, and is incapable of degrading host chromosomal DNA. Infections by this phage will not supply dTMP from host DNA degradation, and should depend on the thymidylate synthase activity present in the cell, as the only source of thymidylate. The dTTP pools were increased somewhat during td8nd28 infections of MB61.21 (the 30-fold thymidylate synthase overproducer), but not in infected wild type cells or MB61.22 (Table XI).

The deoxyribonucleoside UdR can be metabolized to dUMP by phage and E. coli thymidine kinases. Hence, addition of UdR to the growth medium should guarantee a good supply of dUMP for thymidylate synthesis and promote dTTP pool expansion. Surprisingly, addition of UdR to td8nd28 infections had no effect on dTTP pools, and actually seemed to reduce the dTTP pool in td8nd28 infected MB61.21 (Table XI).

The relatively unchanged dTTP pools in the thymidylate synthase

Table X. Thymidylate Synthase Activity in Cells Overexpressing a *Bacillus subtilis*-Related Thymidylate Synthase.

	Relative Thymidylate Synthase Activity	
Cells	A*	B†
E. coli MB45 [HB101(λ*C*$^+$), *thyA*]	-	0 (0.0)
MB49 [HB101(λ*C*$^+$), *thyA*$^+$]	1	1 (1.2)
MB61.21 [HB101(λ*C*$^+$)/pNY10.21]	100	33 (40)
MB61.22 [HB101(λ*C*$^+$)/pNY10.22]	10	3.1 (3.7)

The pNY10.21 and pNY10.22 plasmids constitutively express a *Bacillus subtilis*-related thymidylate synthase. * Values communicated by Marlene Belfort. † Thymidylate synthase activity measured by David Klock of this lab. These values were normalized to MB49 thymidylate synthase activity. The values in parentheses are absolute thymidylate synthase activities in nmols/min/mg protein.

Table XI. dTTP Pools in Cells Overexpressing Thymidylate Synthase.

Cells		pmol dTTP/10^8 Cells
E. coli MB61.21 [HB101(λC^+)/pNY10.21]		20 ± 5
	+ *td8nd28*	43 ± 3
	+ *td8nd28* + UdR (100 μM)	24 ± 7
MB61.22 [HB101(λC^+)/pNY10.22]		14 ± 2
	+ *td8nd28*	20 ± 3
	+ *td8nd28* + UdR (100 μM)	27 ± 1
B		16 ± 4
	+ *td8nd28*	15 ± 1
	+ *td8nd28* + UdR (100 μM)	9 ± 4

The pNY10.21 and pNY10.22 plasmids constitutively express a *Bacillus subtilis*-related thymidylate synthase. The T4 *td8nd28* double mutant is deficient in phage thymidylate synthase (*td8*) and endonuclease II (*nd28*), and should force phage deoxyribonucleotide metabolism to rely on cell or plasmid thymidylate synthase. The *nd28* mutation will prevent introduction of deoxyribonucleotides derived from degradation of the host chromosome. Cells were grown in SM9, and nucleotides were extracted 10 minutes after phage infection (37 °C), by the MeOH/TCA/TOA method.

overexpressors suggests that wild type levels of enzyme are more than sufficient for cell replication and probably do not limit dTMP synthesis under normal growth conditions. However, other steps in nucleotide metabolism, leading up to thymidylate synthase, could be limiting one of the substrates for dTMP biosynthesis, namely dUMP and N^5N^{10}methylenetetrahydrofolate, and prevent dTTP pool expansion. The notion that the MB61.21- or MB61.22-encoded thymidylate synthase is inactive in intact cells, but active in cell extracts, seems unlikely on the basis of two observations. First, the parental cell recipients were thy$^-$, allowing selection for cell transformants in which plasmid-encoded thymidylate synthase was active. Second, in the case of td8nd28-infected MB61.21, the dTTP pools were expanded about 2-fold, indicating the Bacillus subtilis thymidylate synthase can functionally substitute into phage deoxyribonucleotide metabolism. The decrease in MB61.21 dTTP pools on addition of UdR to the cells was unexpected and requires further testing.

G. Deoxyribonucleoside Triphosphate Pools and Mutation Rates in Mutant T4 Gene 1 (dNMP Kinase) and Hypermutator, hm I and hm II, Infections

T4 dNMP kinase is the second enzyme in phage deoxyribonucleotide metabolism required for the synthesis of hmdCTP, and catalyzes the synthesis of dTDP, dGDP, and hmdCDP. Considering its position astride the biosynthetic pathway leading to the synthesis of three deoxyribonucleotides, one can see that mutations in gene 1 (dNMP kinase) could have substantial effects on dNTP pools and, in fact, DNA

replication is inhibited during infections by gene 1 amber mutants.

Since dNMP kinase is an essential gene product, I used a temperature-sensitive gene 1 mutant, tsJW5, to have some control over the degree of enzyme inactivation during phage infections, yet allow some phage growth. This mutant is known to be somewhat leaky for phage production at intermediate temperatures, and, as seen in Table XII, phage growth was not completely inhibited until 40°C. In comparison, growth is halted by 36°C for the T4 gene 42 (dCMP hydroxymethyltransferase) temperature-sensitive mutant, tsLB3 (data not shown).

The influence of the tsJW5 mutation on reversion rates and dNTP pools was measured during infections at 34°C. At this temperature during tsJW5 infections, dGTP pools were somewhat reduced, and dATP and dTTP pools expanded relative to wild type pools (Table XIV). This A/G imbalance, while perhaps not large enough to have a measurable influence on mutagenesis, is in the right direction to stimulate GC-to-AT mutations through increased misincorporation of dAMP for dGMP. In light of this effect on dNTP pools, the slightly increased tsJW5/rIIUV363 AT-to-GC reversion is certainly suggestive, even though the mutation rates measured for both tsJW5 mutants did not meet the minimum 3-fold change required to be considered significant (Table XIII). It should be possible to show this effect more convincingly by measuring mutation rates and dNTP pools for tsJW5/rII mutants at higher temperatures, where larger pool imbalances may exist.

Mutations at the T4 hypermutable locus (hm) have increased spontaneous mutation rates, and are more sensitive to thymineless and base analogue mutagenesis. At the beginning of this study, it was

Table XII. Phage Production of Wild Type and tsJW5 (gene 1)-Infected Cells at Temperatures Permissive and Not Permissive for Phage Growth.

	Phage per Cell			
	Temperature (°C)			
Phage	28°	32°	36°	40°
Wild Type	1 (86.8)	1 (89.4)	0.5 (45.5)	0.4 (34.2)
tsJW5	1 (118)	0.6 (66.6)	0.5 (58)	<0.01 (0.14)

Values in parentheses are phage burst sizes sampled at 30 minutes post-infection. Relative values are normalized to 28 °C bursts.

Table XIII. tsJW5-Stimulated Mutagenesis.

	r11⁻ to r11⁺	
Phage	Reversion Pathway	Reversion Rates x 10^7
r11UV215	AT-to-GC	3.6
tsJW5/r11UV215	AT-to-GC	1.5
r11UV363	GC-to-AT	6.2
tsJW5/r11UV363	GC-to-AT	17

Reversion tests used E. coli BB cells as host cells, and were performed at 34 °C. Reversion rates are the median of three parallel reversion tests.

Table XIV. tsJW5 (gene 1) and Wild Type dNTP Pools.

		pmol dNTP/10^8 cells			
Phage	Time Post-infection	dATP	dTTP	dGTP	hmdCTP(dCTP)
Wild Type	0 min	19	25	6.1	44
	10 min	34	38	33	43
tsJW5	0 min	14	28	11	35
	10 min	92	105	6.4	49

Infections of E. coli B were carried out at 34 °C, and dNTPs extracted by the MeOH/TCA/TOA procedure.

thought that hm mutants mapped in the region of the T4 chromosome near gene 57 and gene 1, and possibly defined a new gene involved in T4 DNA repair or replication. However, it is now believed to map near or in the 3' end of gene 43, which encodes T4 DNA polymerase (J. Drake, personal communication).

In spite of this recent information, the mutagenic activity of hm mutants suggests dTTP pools might somehow be affected. This prediction was not seen in one hm mutant (hm I) dNTP pools, which were virtually identical to wild type pools (Fig. 24). Likewise, hm II dNTP pools, while somewhat reduced, were probably not significantly different from wild type dNTP pools.

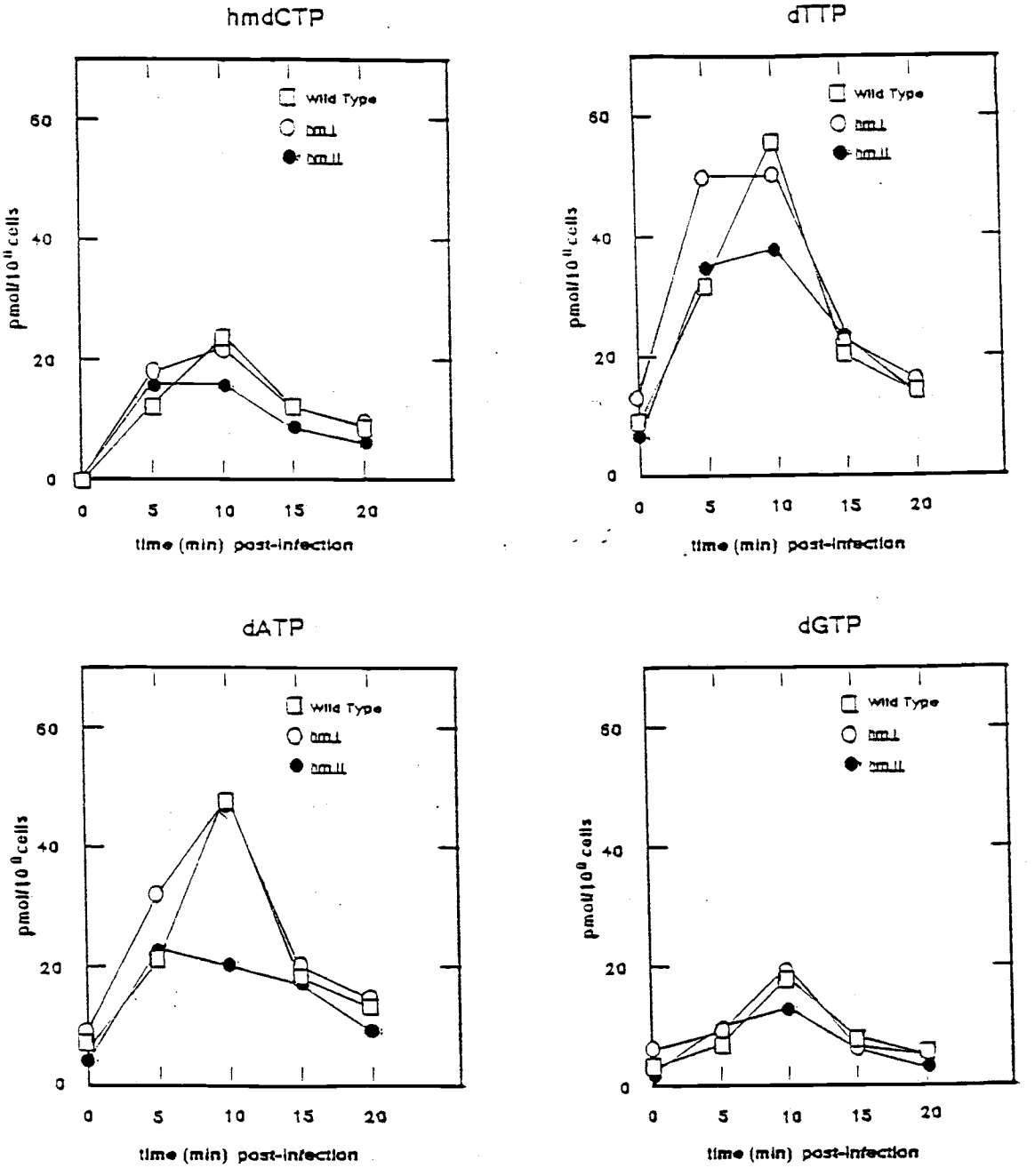


Figure 24. dNTP Pools Measured During T4 Hypermutator (hm) Infections.

IV. DISCUSSION

It is clear, from experiments using reconstructed replication systems *in vitro*, that imbalanced dNTP pools decrease replication fidelity. One advantage in using *in vitro* systems is that dNTP pool concentrations can be carefully controlled and the size of the dNTP imbalance needed to diminish replication fidelity can be precisely established. Furthermore, by using small, well-characterized templates, the mutations caused by dNTP pool imbalances can be determined by DNA sequencing. Finally, the influence that neighboring template sequences have on mutability can be studied by following replication through various sites residing in different sequence environments on the template.

Determinants of replication fidelity *in vivo*, however, are certain to be more complex than mechanisms revealed by studies using *in vitro* replication systems. The identification of other factors controlling cell mutagenesis will probably require investigation at the level of intact cells; hence I was interested in developing an *in vivo* system with the same basic characteristics of the *in vitro* DNA replication system, described above. This report describes the results of my efforts at combining the different genetic and biochemical components necessary to carefully characterize the effects of dNTP pool imbalances on replication fidelity *in vivo*. Since I did not have the same ability to control dNTP concentrations during phage infections as is possible *in vitro*, I manipulated intracellular dNTP pools through a variety of methods, including: (a) mutations in deoxyribonucleotide metabolism, (b) addition of exogenous (deoxy)ribonucleosides, and (c)

poisoning dNTP metabolism with specific antimetabolites. The influence of the dNTP pool imbalance on spontaneous mutation rates was determined by measuring mutation at various well-defined sites in the T4 rII genes; base substitutions or deletions were confirmed by sequencing through the same sites used to measure mutagenesis.

A. Deoxyribonucleotide Metabolism, DNA Replication and dNTP Pools During T4 Infections

The 30- to 40-fold expansion of phage-induced hmdCTP pools and diminished dTTP pools observed during infection with cd phage are comparable to the effects seen by O'Donovan et al. (1971) with mutant E. coli defective in dCTP deaminase, and by Weinberg et al. (1981) with mouse lymphosarcoma cells which have lost dCMP deaminase activity. In both instances, there are marked increases in the intracellular dCTP pools and contracted dTTP pools, when compared with parental cells. Studies measuring the flux of metabolites through deoxyribonucleotide metabolism lead to the estimate that 40% to 80% of dUMP utilized for dTMP biosynthesis is derived from deamination of dCMP by dCMP deaminase in mammalian cells (Jackson, 1978) and bacteriophage T4 (Chiu et al., 1977) and by deamination of dCTP via dCTP deaminase in S. typhimurium and E. coli (Karlström and Larsson, 1967; Neuhard, 1968). It follows that loss of dCMP deaminase activity during T4 infections should block metabolic siphoning of dCMP to dUMP and thus cause expansion of the T4-induced hmdCTP pool. Likewise, loss of dCMP deaminase blocks a major pathway providing substrate for synthesis of dTMP, hence, the diminished dTTP pool observed early

during cd infection should represent the biosynthetic capacity of T4 ribonucleotide reductase alone to provide substrate for thymidylate synthesis. The partial recovery in dTTP pools observed at later times during cd infections might reflect the contribution to dNTP pools of thymidine nucleotides released by T4-encoded nuclease breakdown of host chromosomal DNA, and the subsequent salvage of these deoxyribonucleotides.

The abnormally large intracellular hmdCTP concentration achieved during infections by cd mutant phage should influence deoxyribonucleotide metabolism, and dNTP pools, by altering the regulation of T4 ribonucleotide reductase (see Figure 25 for detailed regulation of T4 ribonucleotide reductase, and Fig. 4 for comparison with E. coli ribonucleotide reductase). Since T4 ribonucleotide reductase is thought to have only one class of binding site for its effectors, two different effectors, when present simultaneously will compete for binding to the enzyme (Berglund, 1972b). I can predict two influences on dNTP pools resulting from hndCTP saturation of effector sites on T4 ribonucleotide reductase. First, since hmdCTP is not the primary effector for rUDP reduction, there should be only 1/2 to 2/3 the rUDP reduction as achieved with the primary effectors of rUDP reduction, rATP and dATP. During infection by cd mutant phage, this net decrease in rUDP reduction will reduce dUMP biosynthesis even further and amplify the hmdCTP/dTTP imbalance initiated by loss of dCMP deaminase. Second, since dATP is an inhibitor of rGDP reduction, hmdCTP saturation of ribonucleotide reductase effector sites should release any residual negative regulation by dATP. Even though hmdCTP is not the primary positive effector for rGDP reduction, loss of dATP

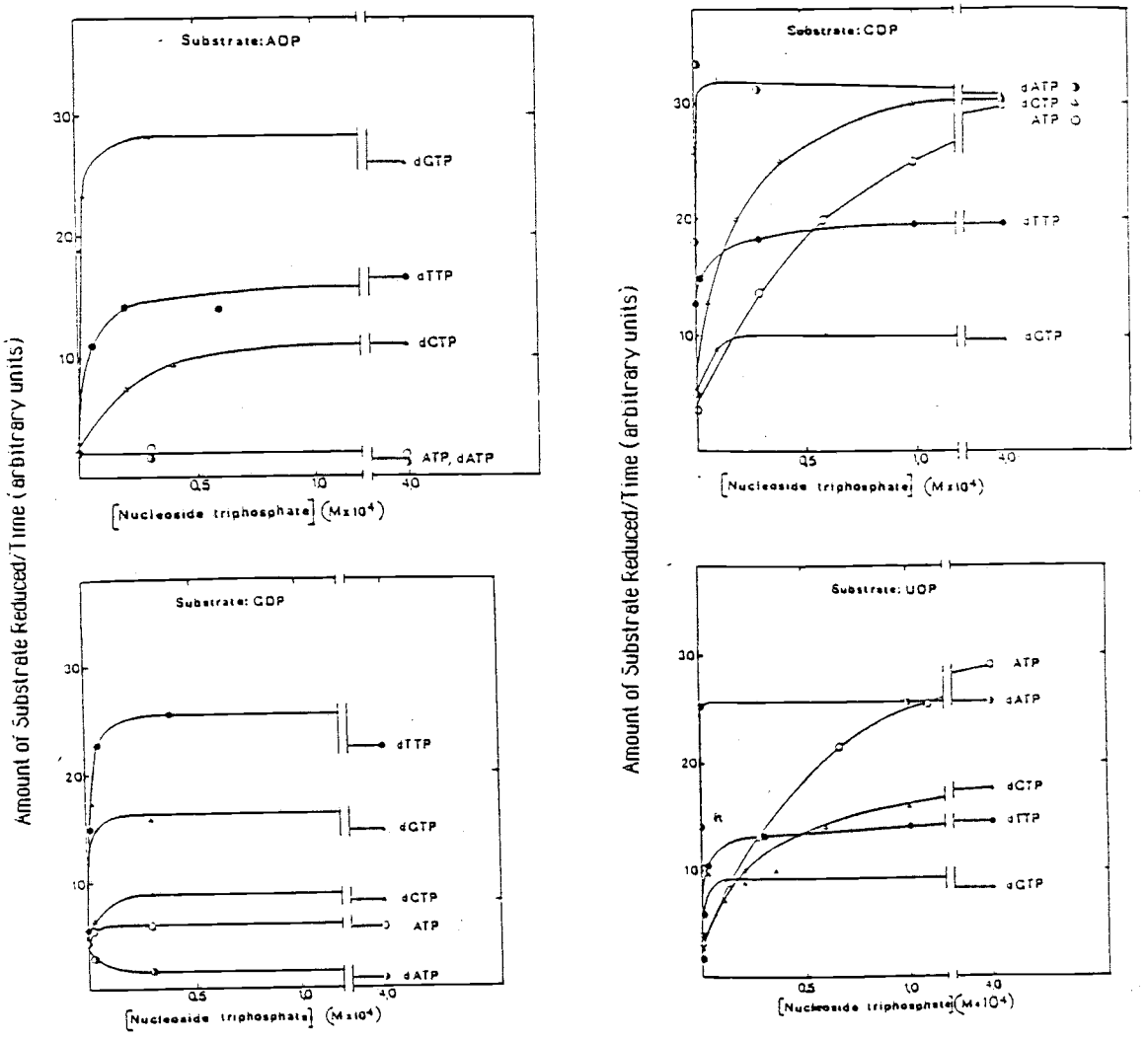


Figure 25. Regulation of T4 Ribonucleotide Reductase by Nucleotide Effectors (from Berglund, 1975b).

inhibition should result in increased reduction of rGDP. Indeed the enlarged dGTP pools (Fig. 18) and diminished rGTP pools (Fig. 19) observed during pseTΔ4(Δcd, ΔpseT) infections are most simply explained by this change in ribonucleotide reductase regulation.

To my surprise, pyrimidine rNTP pools expanded about 8-fold in the dCMP deaminase-deficient pseTΔ4(Δcd, ΔpseT) infections, which represents at least a 4-fold greater accumulation of rUTP and rCTP than seen in pseTΔ5(cd^+, ΔpseT) infections. A similar change in rUTP pools was noted by de Saint Vincent et al. (1980) in mammalian cells also deficient in dCMP deaminase activity. The increase in pyrimidine rNTP pools presents a paradox in the regulation of the de novo pyrimidine pathway, since rCTP is a negative allosteric effector of aspartate transcarbamoylase, one of the initial committed steps in pyrimidine metabolism, and a negative effector of its own biosynthesis by rCTP synthetase (O'Donovan and Neuhard, 1970). Accumulation of rCTP is thus expected to shut down pyrimidine biosynthesis, and may ultimately be responsible for the decrease in pyrimidine rNTP pools observed at 15 to 20 minutes post-infection. In any case, I do not have a ready explanation for the dramatic expansion observed in pyrimidine nucleoside triphosphate pools during cd infections.

I believe that the normalization of hmdCTP and dGTP pools in cd infections (Fig. 21) by the addition of thymidine, can be explained by substrate interactions at yet another site in T4 deoxyribonucleotide metabolism, namely, T4 dNMP kinase. As discussed in the Introduction, dTMP, dGMP, and hmdCMP are all substrates of dNMP kinase, and exhibit competition between each other for binding to the enzyme (Bello and Bessman, 1963). Hence, the enlarged hmdCMP pool in cd infections

should be effective in outcompeting the smaller dTMP pool for dNMP kinase, possibly contributing even further to the hmdCTP/dTTP imbalance. However, with an increasing dTMP pool from addition of thymidine to cd infections (Fig. 21), this competition between hmdCMP and dTMP should shift towards favoring dTMP binding to dNMP kinase, preventing hmdCDP and hmdCTP production, and eventually seen in reduced hmdCTP pools. It is not clear that dGMP is actually metabolized by dNMP kinase during phage infections, since dGTP synthesized by ribonucleotide reductase can bypass this step and be an immediate substrate for the cellular NDP kinase. The thymidine-stimulated reduction of dGTP in cd infections is likely to reflect a more normal state of rGDP reduction by ribonucleotide reductase, once hmdCTP pools have returned to lower concentrations.

Are the dNTP pools measured in pseTΔ5/rIIamHB84 infections sufficient to support wild type rates of T4 DNA replication? The intracellular pseTΔ5/rIIamHB84 dNTP pools are similar to wild type T4-induced deoxyribonucleotide pools measured in these studies, and by Mathews (1972). T4 DNA replication in vitro, using purified phage proteins to replicate the small ΦX174 virus template, reaches replication rates approaching those determined in vivo, at about 200 μM dNTP with a Km of about 75 μM dNTP (Sinha et al., 1980). Assuming that the dNTP pools measured here represent deoxyribonucleotide concentrations at replication forks, and, assuming the intracellular volume of phage-infected cells is about 0.9 femtoliters (Freedman and Krisch, 1971), then by 6 minutes post-infection dATP, dTTP, and hmdCTP concentrations are certainly large enough to support maximal rates of T4 DNA synthesis, as compared to in vitro T4 DNA replication. Phage

DNA replication normally begins at about 5 minutes post-infection and proceeds until cell lysis. Intracellular dGTP pools (Fig. 17, Fig. 18), however, do not reach saturating concentrations until 6 to 11 minutes post-infection and thus might have a role in controlling phage DNA replication through limiting substrate concentration.

Mathews and Sinha (1982) suggested that in phage-infected cells the dNTP concentrations at replication forks are 3-4 times greater than whole cell dNTP concentrations. This proposed localization of dNTPs to the replication fork would surely be to the phage's advantage for rapid establishment of T4 DNA replication early in infection when cell-average dNTP pools are barely adequate to support phage DNA replication. However, dNTP compartmentation might not be required at later times, since the cell-average dNTP pools reported here are large enough soon after infection to support T4 replication. One caveat in drawing these conclusions based on data from experiments using *in vitro* replication systems, is that the *in vitro* T4 replication systems are not routinely used on their native hmC-containing template and probably do not contain all the components of the native replication fork.

B. Deoxyribonucleotide Pools and Mutagenesis

My choice of the T4 *pseTΔ4*(*Δcd*, *ΔpseT*) deletion mutant, used in this study, required testing the influence that loss of polynucleotide kinase had on dNTP pools and mutation rates. The function of polynucleotide kinase during T4 infections of *E. coli* B cells has not been elucidated; however, during infections of a nonpermissive host,

E. coli CTr5x, by *pseT* mutant phage, DNA synthesis is reduced by 50% and late gene expression is shut down (Snyder, 1983). It is well established that some T4 mutator genes are alleles of genes whose products are involved with phage DNA replication (Bernstein, 1971; Bernstein et al., 1972; Koch et al., 1976; Speyer et al., 1966), hence, my concern that loss of polynucleotide kinase in itself could result in a mutator phenotype or altered dNTP pools. The data indicated that loss of polynucleotide kinase, during *pseTΔ5*(*cd*$^+$, *ΔpseT*) infections, resulted in unchanged dNTP pools (Fig. 18) or mutation rates (Table V) when compared to wild type or *rII*-single infections.

While the additional loss of polynucleotide kinase in *pseTΔ4*(*Δcd*, *ΔpseT*) infections could influence reversion rates, I do not believe its absence is necessary to explain the increased AT-to-GC reversion seen in Table V. This conclusion is supported by the following observations. First, as mentioned above, loss of polynucleotide kinase does not affect reversion of any of the *rII* mutants used (Table V). Secondly, the loss of dCMP deaminase activity alone, during *cdN16* (*cd*$^-$, *pseT*$^+$)/*rIIUV215* infections, results in comparable AT-to-GC mutation rates (Table VII) and dNTP pool imbalances (data not shown) as seen in *pseTΔ4*(*Δcd*, *ΔpseT*)/*rIIUV215* infections.

Inactivation of two other enzymes in T4 deoxyribonucleotide metabolism, thymidylate synthase (Drake and Greening, 1970; Bernstein et al., 1972; Smith et al., 1973) and dCMP hydroxymethyltransferase (Chiu and Greenberg, 1973; Williams and Drake, 1977), are known to affect phage mutation rates. Comparable to those examples, the increased AT-to-GC reversion rates seen in *pseTΔ4* and *cdN16*

infections are most likely caused by imbalanced dNTP pools. Whereas the extreme hmdCTP/dTTP imbalance clearly promotes A:C mispairing, the moderate increase in dGTP pools, and essentially unchanged dATP pools, should not substantially change the number of G:T mispairs already occurring.

The allele-specific response of thymidine-influenced mutation rates during pseTΔ4 and cdN16 infections might reflect a difference in dNTP balance in the micro-environment around the replication forks of these two mutants. If the replication fork is somewhat "inaccessible" to the free dNTP pools in the cell, then the free dNTP pools, and any imbalances therein, would not influence base misincorporations to the same degree as an "accessible" (to free dNTPs) replication fork. Since dCMP deaminase is thought to be an integral component of the T4 dNTP complex, and considering its pivotal role in controlling dCMP and dTMP levels in phage infections, I felt that the physical loss of dCMP deaminase, during infections by the pseTΔ4 mutant, might disrupt the dNTP complex more completely than the cdN16 missense mutant, and create an "accessible" replication fork. This model is an attractive explanation for the observation that AT-to-GC mutation rates are somewhat reduced with normalization of pseTΔ4 dNTP pools on addition of thymidine, but not with cdN16. However, the cdN16 dNTP pool response to thymidine has not yet been determined. Since there is other evidence interpreted as illustrating protein-protein interactions between the T4 dNTP complex and the replication complex (Chao et al., 1977; Macdonald and Hall, 1984), it will ultimately be necessary to show that any effects on DNA replication, and mutagenesis, are dNTP pool-dependent, and not caused by reduced

replication fidelity from altered protein-protein interactions.

C. Template Effects on Mutagenesis

The two most apparent template-specific effects on reversion were seen in the almost exclusive recovery of rIIamHB74$^+$ (TGG) revertants (Table VI) and the remarkably high rIIUV215 AT-to-GC reversion (Table V). In principle, because the rIIamHB74 mutation maps into the rIIB dispensable region: (1) reversion to any nonterminating rIIamHB74$^+$ codon should be tolerated by the rIIB protein; and (2) there should not be any selection for or against any particular rIIamHB74$^+$ revertants. AT-to-GC transitions at the second position of amber codons also predominate in aminopurine mutagenesis of replicating T4 (Koch, 1971; Ronen et al., 1976), and with in vitro replication systems using purified T4 replication proteins, promoting AT-to-GC transitions by deoxyribonucleotide pool imbalances (Sinha and Goodman, 1983). Why then do TGG revertants seem to occur more frequently than CAG revertants, when both can be generated by either A:C or G:T mispairs (Fig. 26)? Misincorporated nucleotides at the first position of the rIIamHB74 amber codon are bound by thermodynamically less stable neighboring A:T base pairs, while reversion at the second position has one G:C base pair as a nearest neighbor (Fig. 26), and in fact would be part of a short G:C run. If rIIamHB74 reversion is driven by misincorporation of hmdCMP into DNA, which seems likely considering the pseTΔ4 dNTP pool imbalances, the misinsertion event must be during the replicative pathway illustrated in Figure 26c, and thus suggests that in this case neighboring G:C bases may play a role

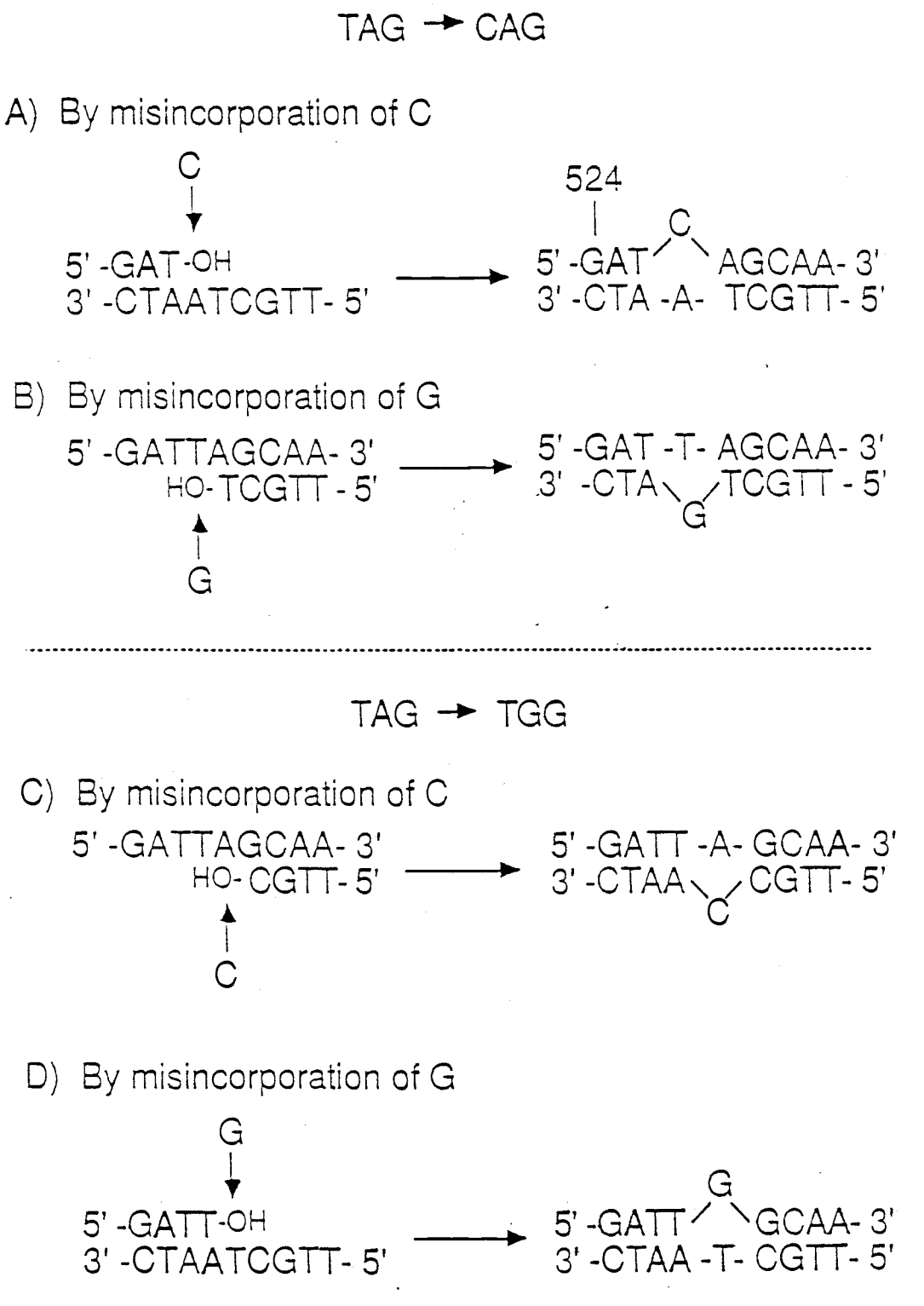


Figure 26. AT-to-GC Reversion Pathways in the rIIamHB74 Codon.

in determining the frequency of misinsertion.

In the pseTΔ4(Δcd, ΔpseT) and cdN16 backgrounds, rIIUV215 AT-to-GC reversion was about 100 times greater than any of the other AT-to-GC reverting mutants, rIIamHB74, rIIamHB84 or rIIHD263. The rIIUV215 mutant also exhibits similar high mutation frequencies under conditions that limit thymidylate biosynthesis by mutations in the T4 structural gene encoding thymidylate synthase or poisoning thymidylate synthase activity with the thymidylate analog, 5-fluorodeoxyuridylate (Smith et al., 1973). One model, originally suggested by Bessman and Reha-Krantz (1977) to explain high reversion rates of the rIIUV373 mutant, and which may apply to rIIUV215 reversion, is that high mutability is associated with increased DNA-duplex stability in G+C rich regions of DNA. I propose that the rIIUV215 mutation resides in a G+C rich region and that the dNTP pools induced during T4 cd infections might dispose G+C rich regions to increased mutability, for the following reasons. First, when suboptimal dNTP concentrations are used to support DNA replication in vitro, essentially decreasing the polymerase to exonuclease ratio, the likelihood of editing by the 3'-5' exonuclease of DNA polymerase increases (Galas and Branscomb, 1978; Clayton et al., 1979). The enlarged hmdCTP and dGTP pools induced during cd infections should ensure efficient replication through G+C rich regions in the T4 genome, promoting replication to proceed past misincorporation sites and, by maintaining a high polymerase to exonuclease ratio, suppress editing by 3'-5' exonuclease activity. Additionally, as mentioned above, increased DNA-duplex stability in G+C-rich sequences will contribute to minimize DNA polymerase exonucleolytic editing by impeding the polymerase's ability

to peel back previously synthesized sequences, in order to remove mismatched residues (Galas and Branscomb, 1978; Petruska and Goodman, 1985). To find evidence in support of this hypothesis requires sequencing into the region surrounding the rIIUV215 mutation.

Transition mutagenesis can be partitioned into two pathways that use either A:C or G:T mispaired intermediates. As mentioned above, with in vitro replication systems, dNTP pool imbalances that promote G:T mispairs result in higher mutation rates than imbalances promoting A:C mispairs. However, to my knowledge, this has not been described for mutagenesis in vivo. One explanation, then, for the assorted mutation rates measured at different mutant rII sites, is that template effects might predispose mutation by A:C intermediates at one site and G:T intermediates at another. In this sense, rIIamHB74 reversion, discussed above, could be considered an example of local template structure predisposing A:C intermediates at the second nucleotide of the amber codon. This partitioning of mutagenic pathways, if sensitive to dNTP pool imbalances, can be tested by inducing a dGTP/dATP imbalance during phage infection, that is comparable to the hmdCTP/dTTP imbalance measured in pseTΔ4 infections. I expect to observe two phenomena from this experiment. First, during dGTP/dATP pool imbalances I would expect some rII sites, those predisposed to mutation by G:T intermediates, to have higher mutation rates than sites reverting by A:C intermediates. Secondly, if G:T mispairs are more frequently formed than A:C intermediates in vivo, mutagenesis during smaller dGTP/dATP pool imbalances should be equal to or higher than, mutagenesis by hmdCTP/dTTP imbalances at a specific basepair (for example the second nucleotide of rIIamHB74.

D. Ribonucleotide Reductase and BrUdR Mutagenesis

It is clear, seen in the rescue of phage production during T4 nrdBamB55 infections of ED8689/pPS2, that E. coli ribonucleotide reductase can functionally complement the loss of T4 ribonucleotide reductase. It is not known whether this is accomplished simply through overcoming a bottleneck in deoxyribonucleotide metabolism and replenishing the supply of dNTPs, or whether it also involves physical substitution of E. coli ribonucleotide reductase into the phage dNTP complex. The measurement of dNTP pools during nrdBamB55 infections will help answer this question.

However, from my available data it seems unlikely that substitution of E. coli ribonucleotide reductase for that of T4 will result in dramatically altered dNTP pools. This prediction is based on two observations. First, the relative dNTP concentrations measured in uninfected E. coli are not dramatically different from those seen in wild type T4 infections (Fig. 17, Fig. 19) late during phage infections. Second, from observations of other researchers measuring mutation rates during conditions of dNTP pool imbalance, and from my experience with phage mutagenesis and altered dNTP pools during cd$^-$ infections, the mutagenic pathway stimulated usually reflects the pool imbalance. T4 mutation rates were weakly stimulated, in a nrdBamB55/rII background, and showed no preference for AT-to-GC or GC-to-AT mutagenesis during infections of ED8689/pPS2. Alternatively, if E. coli ribonucleotide reductase is physically incorporated into the T4 dNTP complex, the ambivalent mutagenesis measured during nrdBamB55/rII infections could be from altered protein-protein

interactions, like those postulated for mutant gene 42 infections (Williams and Drake, 1977).

As mentioned above, BrUdR-stimulated mutagenesis induces both AT-to-GC and GC-to-AT transitions. This is believed to be brought about by the ability of bromouracil to assume a tautomeric or ionized form that allows mispairing of BrU residues with guanines. This mispairing can occur after BrUdR-nucleotides have been incorporated into DNA (errors of replication), or incoming BrUdR-nucleotides can be misincorporated opposite template guanines (errors of incorporation). These two mechanisms, and the mutations induced, are illustrated in Figure 27.

According to the model proposed by Hopkins and Goodman (1980), the transition pathway stimulated by BrUdR mutagenesis depends essentially on the BrdUTP/dCTP vs BrdUTP/dTTP ratio at the replication fork. During T4 infections, the preference for GC-to-AT transitions stimulated during BrUdR mutagenesis suggests that BrdUTP competition with hmdCTP for incorporation into T4 DNA is more important than BrdUTP competition with dTTP, while in E. coli, increased AT-to-GC mutagenesis suggests that BrdUTP is competing more with dTTP. Hopkins and Goodman (1982) went on to suggest that the ratio of BrdUTP to other nucleotides might be determined by enzymes in the organism's deoxyribonucleotide metabolic pathway, and suggested that ribonucleotide reductase and thymidine kinase might be involved in establishing the levels of these dNTPs. Since the ribonucleotide reductases of T4 and E. coli are regulated differently by dNTPs, I felt that exchanging ribonucleotide reductases between T4 and E. coli metabolism would provide a handle for manipulating BrdUTP/hmdCTP and

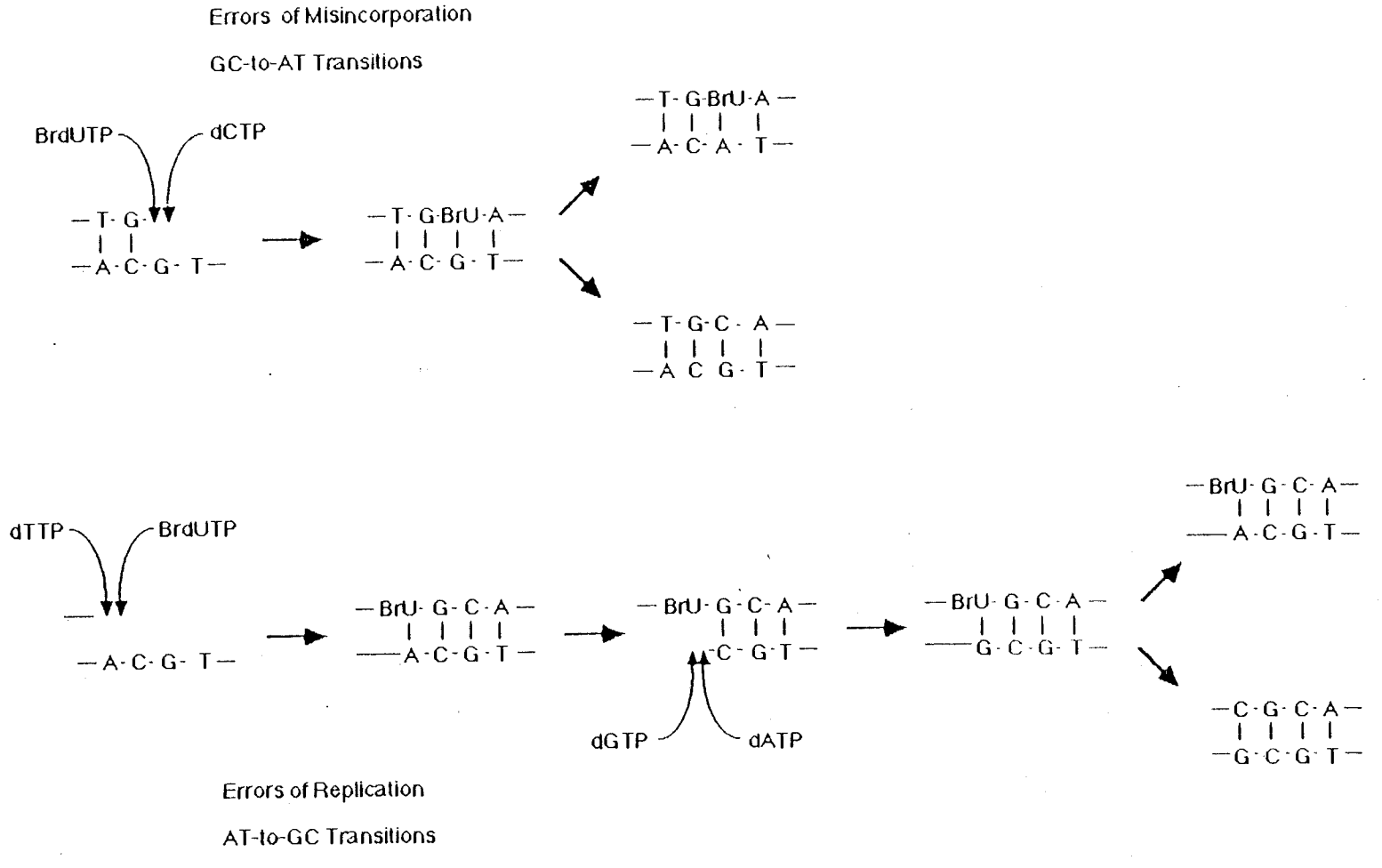


Figure 27. BrdUTP Mutations Induced by Errors of Incorporation or Errors of Replication.

BrdUTP/dTTP ratios, and BrUdR mutagenesis, during phage infections. In fact, substitution of T4 ribonucleotide reductase by E. coli ribonucleotide reductase appears to cause BrUdR-stimulated mutagenesis to favor AT-to-GC transitions over GC-to-AT transitions during phage infections (Table IX and Fig. 23).

When considering only the properties and regulation of E. coli or T4 ribonucleotide reductase, it is difficult to explain these results in terms of dNTP pools, and the effects of BrUdR addition thereon. Instead, one must consider the entire deoxyribonucleotide metabolic pathway, and its regulation by intracellular BrdUTP pools in establishing a BrdUTP/dTTP or BrdUTP/dCTP ratio that will favor either AT-to-GC or GC-to-AT transitions.

During wild type T4 infections, I propose that dNMP kinase is the site in deoxyribonucleotide metabolism whereby BrUdR, or rather BrdUMP, first acts to establish a BrdUTP/hmdCTP ratio stimulating GC-to-AT transitions. In this model, T4 ribonucleotide reductase and dCMP deaminase play only a supporting role in establishing the BrdUTP/hmdCTP balance, by maintaining the 2:1 ratio of dTMP:hmdCMP synthesized during phage infections. This is a reasonable expectation since reduction of pyrimidine ribonucleotides by purified T4 ribonucleotide reductase is not inhibited by dTTP, or probably its analog BrdUTP. And, since dCDP synthesis is maintained by the phage ribonucleotide reductase, which should result in relatively normal intracellular hmdCTP concentrations, in vivo phage dCMP deaminase activity will be less inhibited by increasing BrdUTP pools. This is an important prediction, as phage dCMP deaminase, and cell dCTP deaminase, each has a key role in maintaining dUMP synthesis, and

eventually dTTP synthesis. Both enzymes are regulated by the ratio of dCTP/dTTP present, and conditions maintaining relatively high (or even normal) intracellular dCTP concentrations should help to maintain dUMP biosynthesis, even in the face of increasing dTTP or BrdUTP pools. To recapitulate, hmdCMP and dTMP synthesis, in T4-infected cells, should be relatively undisturbed by moderate concentrations of BrUdR-derived deoxyribonucleotides.

One clue in identifying dNMP kinase as the putative target in T4 metabolism where the BrdUTP/hmdCTP balance is set, can be seen by comparing the metabolic routes leading to hmdCTP (and dCTP), dTTP and BrdUTP in uninfected and T4 infected E. coli (Fig. 28). With the above considerations about the role of T4 ribonucleotide reductase and dCMP deaminase, it is obvious that the only step in phage metabolism common to all three nucleotides, and the only step where BrdUMP can directly influence hmdCTP synthesis, is at dNMP kinase. This will allow the relative proportions of hmdCDP, dTDP, and BrdUDP synthesized by dNMP kinase to be set by competition between hmdCMP, dTMP, and BrdUMP. The ratio of BrdUTP/hmdCTP will essentially be proportional to the amount of BrUdR in the medium.

Another way to examine the effects of BrdUMP on T4 pyrimidine deoxyribonucleotide pools is to consider a scenario whereby the intracellular BrdUMP concentration is equal to the intracellular dTMP pool. Since dTMP and hmdCMP synthesis should be relatively undisturbed, and synthesized in their characteristic 2:1 ratio, the presence of BrdUMP will bring about pyrimidine dNMP pools with a ratio of BrdUMP:dTMP:hmdCMP = 2:2:1. The ratio between these three dNTPs should essentially be the same as the dNMP ratio, and the dTTP and

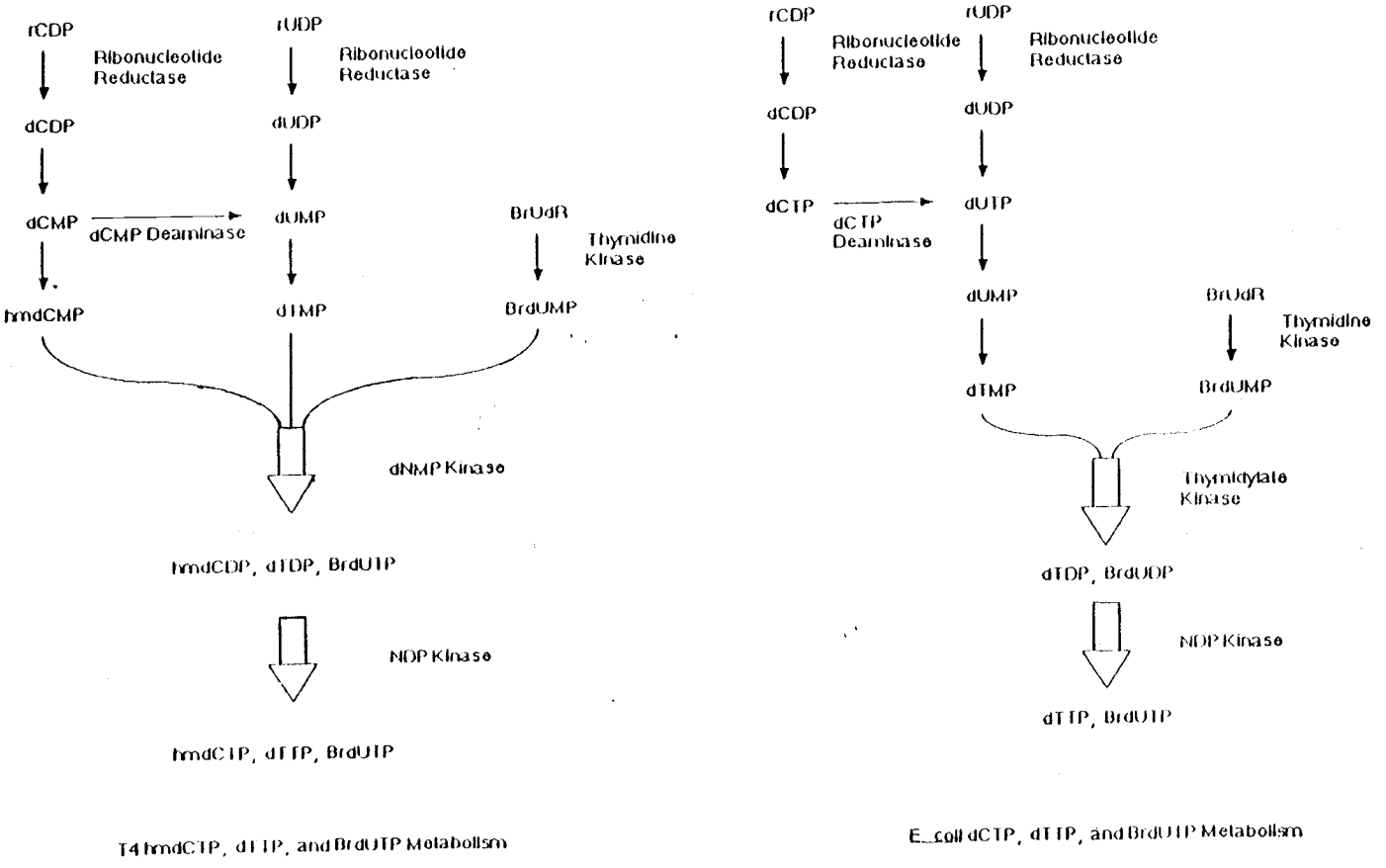


Figure 28. BrUdR Metabolism in T4-Infected and Uninfected E. coli.

hmdCTP pools both will be diluted proportionately to the BrdUMP (and BrUdR) concentration. However, hmdCTP is now represents only 20% of the total pyrimidine dNTP pool, as compared to 50% in the absence of BrdUMP.

Modeling the response of uninfected E. coli deoxyribonucleotide metabolism to BrdUTP levels is more speculative than with T4 metabolism. In contrast to T4, there are two allosterically regulated enzymes in uninfected E. coli, namely, ribonucleotide reductase and dCTP deaminase, that will coordinately determine the amount of dCTP and dTMP synthesized. Moreover, since BrdUTP is influencing dCTP and dTMP synthesis in uninfected E. coli by regulation of enzyme activities, rather than by substrate competition, I expect a greater disparity between the relative concentrations of dCTP, dTTP, and BrdUTP than seen in T4-infected cells.

If we first follow the pathway leading to dCTP in uninfected E. coli, it is a straightforward prediction that large BrdUTP pools will inhibit pyrimidine dNDP synthesis by E. coli ribonucleotide reductase and bring about a decrease in dCTP pools (Thelander and Reichard, 1979). In contrast to T4, there are not any other steps in E. coli metabolism unique to pyrimidine synthesis, where BrUdR-nucleotides can compete with deoxycytidine nucleotides. For example, as with T4 deoxyribonucleotide metabolism, dCTP, as well as all other dNTPs, is synthesized by NDP kinase. Furthermore, dCDP can be synthesized through the salvage of dCMP by CMP/dCMP kinase. Since this enzyme does not use dUMP or dTMP as substrates, I do not expect BrdUMP to be utilized by this enzyme. Simply put, in E. coli the step exerting the greatest influence on dCTP pools will be regulation of ribonucleotide

reductase by intracellular BrdUTP pools.

In contrast to T4, there are three steps in the E. coli metabolic pathway leading to dTTP that should be affected by BrUdR-derived nucleotides. (1) Since about 75% of the cell's dUMP is synthesized via dCTP deaminase (Karlström and Larsson, 1967; Neuhard, 1968), any reduction in dCTP pools, from inhibition of ribonucleotide reductase by BrdUTP, should be paralleled by a reduction in dUMP pools. In contrast to the situation in T4, the reduced dCTP pools and high BrdUTP pools in uninfected E. coli should cause inhibition of dCTP deaminase, and reduce dUMP biosynthesis even further. The regulation of dCTP deaminase activity by deoxyribonucleotides is comparable to that of dCMP deaminase, whereby dCTP is a positive effector of enzyme activity, and dTTP, and almost certainly BrdUTP, are inhibitors. (2) The overall inhibition of pyrimidine reduction by ribonucleotide reductase as a consequence of high BrdUTP pools, will result in less dUDP production and decrease dUMP pools originating from this source. (3) Since BrdUMP and dTMP are both substrates for thymidylate kinase, competition between these two substrates will dilute the amount of dTTP synthesized even further, increasing the BrdUTP/dTTP ratio at the replication fork.

The situation with respect to BrUdR-stimulated AT-to-GC mutation rates during nrdBamB55/rII infections is perfectly analogous to the effects of BrUdR on E. coli. Addition of BrUdR to nrdBamB55/rII-infected ED8689/pPS2 ribonucleotide reductase-overproducing cells, will cause inhibition of dCDP and dUDP synthesis by ribonucleotide reductase. I believe that under these conditions, namely, a large BrdUTP pool and decreased hmdCTP pool, T4 dCMP deaminase activity will

be inhibited comparably to E. coli dCTP deaminase. The net effect should be (1) severely decreased intracellular dTTP and hmdCTP pools, similar to the situatioin in E. coli, and (2) an hmdCMP pool larger than the corresponding dTMP pool. This hmdCMP/dTMP imbalance prior to dNMP kinase will establish synthesis of a larger ratio of BrdUTP/dTTP than BrdUTP/hmdCTP. These effects of BrUdR-nucleotides on T4, E. coli, and T4-nrd mutant metabolism and dNTP pools are summarized in Table XV.

In this model of BrUdR mutagenesis, I have discussed how specific differences in the deoxyribonucleotide metabolic pathways of T4 and E. coli can lead to characteristic dNTP pool ratios and mutagenic pathways. It is unlikely that pool effects are the only mechanism determining the preference for BrUdR-induced AT-to-GC or GC-to-AT transitions, especially when one considers there are fundamental differences between T4 and E. coli DNA replication systems, and the fact that E. coli has more extensive and complex DNA repair systems than T4. In fact, one group has suggested that BrUdR mutagenesis in E. coli is largely a consequence of repairing BrU lesions in DNA, or lesions originating with BrU incorporation (Szyszko et al., 1983; Pietrzykowska et al., 1983; Pietrzykowska et al., 1985). Another factor that must be eventually taken into account, with regard to the utilization of bromouracil and perhaps BrUdR as substrates for DNA replication, is that bromouracil is less efficiently utilized and incorporated into E. coli DNA as compared to thymine (Kanner and Hanawalt, 1968; Rydberg, 1977). This is especially important when comparing the BrUdR-pool effects of two different organisms.

One question that has not been answered is what ratios of

Table XV. Proposed Effects of BrUdR on T4 and *E. coli* dNTP pools.

Proposed BrUdR Effects on T4 and *E. coli* dNTP pools

	T4 *nrd*$^+$	*E. coli*	T4 *nrd*$^-$-Infected ED8689/pPS2
Observed BrUdr Stimulated Mutagenesis	GC-to-AT	AT-to-GC	AT-to-GC
Target Enzymes of BrUdR Nucleotides	dNMP Kinase	Ribonucleotide Reductase dCTP Deaminase Thymidylate Kinase	Ribonucleotide Reductase dCMP Deaminase dNMP Kinase
BrdUTP Effects on dNTP Pools	Small to moderate reduction in hmdCTP and dTTP pools	Large reduction in dCTP and dTTP pools relative to wild type T4 pools	Large reduction in dCTP and dTTP pools relative to wild type T4 pools
BrdUTP/(hm)dCTP	Small to moderate	Large	Large
BrdUTP/dTTP	Small to moderate	Large	Large
(hm)dCTP/dTTP	≈1:2	≥1	≥1

BrdUTP/dCTP and BrdUTP/dTTP stimulate AT-to-GC or GC-to-AT transitions in the cell? A natural prediction is that at low, but increasing, ratios of BrdUTP/dCTP mostly GC-to-AT transitions will occur, and eventually with increasing BrdUTP pools, the BrdUTP/dTTP balance will come to dominate, as will the incidence of AT-to-GC transitions. Measurement of dTTP, hmdCTP, and BrdUTP pools during nrd$^-$, and nrd$^+$ phage infections are underway to test this prediction.

V. BIBLIOGRAPHY

Aronow, B., Watts, T., Lassetter, J., Washtien, W., and Ullman, B. (1984) J. Biol. Chem. 259, 9035-9043.

Averett, D.R., Lubbers, C., Elion, G.B., and Spector, T. (1983) J. Biol. Chem. 258, 9831-9838.

Barnett, L., Brenner, S., Crick, F.H.C., Shulman, R.G., and Watts-Tobin, R.J. (1967) Phil. Trans. of the Royal Soc. London series B 252, 487-537.

Bello, L.J. and Bessman, M.J. (1963) J. Biol. Chem. 238, 1777-1787.

Benzer, S. (1961) Proc. Natl. Acad. Sci. U.S.A. 47, 403-415.

Berglund, O., Karlström, O., and Rèichard, P. (1969) Proc. Natl. Acad. Sci. U.S.A. 62, 2735-2739.

Berglund, O. (1972a) J. Biol. Chem. 247, 7270-7275.

Berglund, O. (1972b) J. Biol. Chem. 247, 7276-7281.

Berglund, O. (1975) J. Biol. Chem. 250, 7450-7455.

Bernstein, H. (1971) J. Virol. 7, 460-466.

Bernstein, C., Bernstein, H., Mufti, S. and Strom, B. (1972) Mutation Res. 16, 113-119.

Bernstein, C., and Wallace, S.S. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 138-151, American Society for Microbiology, Washington, D.C.

Bertani, L.E., Häggmark, A., and Reichard, P. (1963) J. Biol. Chem. 238, 3407-3413.

Bessman, M.J., Muzyczka, N., Goodman, M.F., and Schnaar, R.L. (1974) J. Mol. Biol. 88, 409-421.

Bessman, M.J. and Reha-Krantz, L.J. (1977) J. Mol. Biol. 116, 115-123.

Berger, H., and Pardoll, D. (1976) J. Virol. 20, 441-445.

Bradley, M.O., and Sharkey, N.A. (1978) Nature 274, 607-608.

Bresler, S.E., Mosevitsky, M.I., Vyacheslavov, L.G. (1973) Mutat. Res. 19, 281-293.

Bridges, B.A., Law, J., and Munson, R.J. (1968) Molec. Gen. Genet. 103, 266-273.

Brody, E., Rabussay, D., and Hall, D.H. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 174-183, American Society for Microbiology, Washington, D.C.

Capco, G.R., Krupp, J.R., and Mathews, C.K. (1973) Arch. Biochem. Biophys. 158, 726-735.

Chao, J., Leach, M., and Karam, J. (1977) J. Virol. 24, 557-563.

Chen, S.-C., Brown, P.R., and Rosie, D.M. (1977) J. Chromat. Science 15, 218-221.

Chiu, C.-S., and Greenberg, G.R. (1973) J. Virol. 12, 199-201.

Chiu, C.-S., Ruettinger, T., Flanegan, J.B., and Greenberg, G.R. (1977) J. Biol. Chem. 252, 8603-8608.

Christensen, A.C., and Young, E.T. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 184-188, American Society for Microbiology, Washington, D.C.

Clayton, L.K., Goodman, M.F., Branscomb, E.W., and Galas, D.J. (1979) J. Biol. Chem. 254, 1902-1912.

Cohen, G.H. (1972) J. Virol. 9, 408-418.

Conkling, M.A., Koch, R.E., and Drake, J.W. (1980) J. Mol. Biol. 143, 303-315.

Coukell, M.B. and Yanofsky, C. (1970) Nature 228, 633-635.

Crick, F.H.C., Barnett, L., Brenner, S., and Watts-Tobin, R.J. (1961) Nature (London) 192, 1227-1232.

Davis, R.W., Botstein, D., and Roth, J.R. (1980) A Manual for Genetic Engineering: Advanced Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

de Boer, J.G., and Ripley, L.S. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 5528-5531.

de Saint Vincent, B.R., De'champs, M., and Buttin', G. (1980) J. Biol. Chem. 255, 162-167.

de Serres, F.J., de. (1985) Genetic Consequences of Nucleotide Pool Imbalances, Plenum Press, New York.

de Vries, F.A.J., Swart-Idenburg, C.J.H., and de Waard, A. (1972) Mol. Gen. Genet. 117, 60-71.

DeVries, J.K., and Wallace, S.S. (1982) Molec. Gen. Genet. 186, 101-105.

Drake, J.W. and Allen, E.F. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 339-344.

Drake, J.W. (1970) The Molecular Basis of Mutation, Holden-Day,

Drake, J.W. and Greening, E.O. (1970) Proc. Natl. Acad. Sci. U.S.A. 66, 823-834.

Drake, J.W. (1973) Genetics 73, 45-64.

Drake, J.W. and Ripley, L.S. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 312-320, American Society for Microbiology, Washington, D.C.

Erikson, J.S. (1971) Ph.D. Thesis, Univ. Arizona.

Erikson, J.S. and Mathews, C.K. (1971) Biochem. Biophys. Res. Comm. 45, 1164-1170.

Fersht, A.R. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4946-4950.

Flaks, J.G. and Cohen, S.S. (1959) J. Biol. Chem. 234, 2981-2986.

Flanegan, J.B. and Greenberg, G.R. (1977) J. Biol. Chem. 252, 3019-3027.

Freese, E. (1959) J. Mol. Biol. 1, 87-105.

Freedman, M.L., and Krisch, R.E. (1971) J. Virol. 8, 87-94.

Galas, D.J., and Branscomb, E.W. (1978) J. Mol. Biol. 124, 653-687.

Geiger, J.R., and Speyer, J.F. (1977) Molec. Gen. Genet. 153, 87-97.

Geiduschek, E.P., Elliot, T., and Kassavetis, G.A. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 189-192, American Society for Microbiology, Washington, D.C.

Gillin, F.D., and Nossal, N.G. (1976) J. Biol. Chem. 251, 5219-5224.

Goodman, M.F., Gore, W.C., Muzyczka, N., and Bessman, M.J. (1974) J. Mol. Biol. 88, 423-435.

Green, R.R., and Drake, J.W. (1974) Genetics 78, 81-89.

Hall, D.H. and Tessman, I. (1966) Virology 29, 339-345.

Hall, D.H. (1967) Proc. Natl. Acad. Sci. U.S.A. 58, 584-591.

Hall, D.H., Tessman, I., and Karlström, O. (1967) Virology 31, 442-448.

Hall, J.D., Coen, D.M., Fisher, B.L., Weisslitz, M., Randall, S., Almy, R.E., Gelep, P.T., and Schaffer, P.A. (1984) Virology 132, 26-37.

Hall, R.M. and Brammer, W.J. (1973) Molec. Gen. Genet. 121, 271-276.

Hershey, A.D. (1946) Genetics 31, 620-640.

Hershfield, M.S. (1973) J. Biol. Chem. 248, 1417-1423.

Hibner, U., and Alberts, B.M. (1980) Nature (London) 285, 300-305.

Holmes, A.J., and Eisenstark, A. (1968) Mutat. Res. 5, 15-21.

Hopkins, R.L., and Goodman, M.F. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1801-1805.

Howard, B.D., and Tessman, I. (1964) J. Mol. Biol. 9, 364-371.

Hunting, D. and Henderson, J.F. (1981) Can. J. Biochem. 59, 723-727.

Hunting, D. and Henderson, J.F. (1982) Meth. Canc. Res. 20, 245-284.

Ingraham, J.L., and Ginther, C.L. (1978) Meth. Enz. 51, 371-375.

Jackson, R.C. (1978) J. Biol. Chem. 253, 7440-7446.

Johnson, J.R. and Hall, D.H. (1973) Virology 53, 413-426.

Kanner, L., and Hanawalt, P. (1968) Biochim. Biophys. Acta 157, 532-545.

Karlström, O., and Larsson, A. (1967) Eur. J. Biochem. 3, 164-170.

Khym, J.X. (1975) Clinical Chem. 21, 1245-1252.

Koch, R.E. (1971) Proc. Natl. Acad. Sci. U.S.A. 68, 773-776.

Koch, R.E., McGaw, M.K., and Drake, J.W. (1976) J. Virol. 19, 490-494.

Kozloff, L.M. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 25-31, American Society for Microbiology, Washington, D.C.

Kozinski, A.W. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 111-119, American Society for Microbiology, Washington, D.C.

Kreuzer, K.N., and Alberts, B.M. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 3345-3349.

Kreuzer, K.N., and Alberts, B.M. (1986) J. Mol. Biol. 188, 185-198.

Kunz, B.A. (1982) Env. Mutag. 4, 695-725.

Kunz, B.A., and Glickman, B.W. (1985) J. Bacteriol. 162, 859-864.

Kutter, E.M., and Wiberg, J.S. (1968) J. Mol. Biol. 38, 395-406.

Kutter, E.M. and Snyder, L. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 56-57, American Society for Microbiology, Washington, D.C.

Larsson, A. and Reichard, P. (1966a) J. Biol. Chem. 241, 2533-2539.

Larsson, A. and Reichard, P. (1966b) J. Biol. Chem. 241, 2540-2549.

Lo, K.-Y., and Bessman, M.J. (1976a) J. Biol. Chem. 251, 2475-2479.

Lo, K.-Y., and Bessman, M.J. (1976b) J. Biol. Chem. 251, 2480-2486.

Luria, S.E., Darnell, Jr., J.E., Baltimore, D., and Campbell, A. (1978) General Virology 3rd ed. John Wiley and Sons.

Lyons, S.M., Speyer, J.F., and Schendel, P.F. (1985) Molec. Gen. Genet. 198, 336-347.

Macdonald, P.M. and Hall, D.H. (1984) Genetics 107, 343-353.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning, Cold Spring Harbor Laboratory; Cold Spring Harbor, New York.

Mathews, C.K. (1965) J. Bacteriol. 90, 648-652.

Mathews, C.K. (1967a) J. Biol. Chem. 242, 4083-4086.

Mathews, C.K. (1967b) J. Virol. 1, 963-967.

Mathews, C.K. (1971) Bacteriophage Biochemistry, Van Nostrand Reinhold Co., New York, New York.

Mathews, C.K. (1972) J. Biol. Chem. 247, 7430-7438.

Mathews, C.K., and Sinha, N.K. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 302-306.

Mathews, C.K., and Allen, J.R. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 59-70, American Society for Microbiology, Washington, D.C.

McPheeters, D.S., Christensen, A., Young, E.T., Stormo, G., and Gold, L. (1986) Nucleic Acids Res. 14, 5813-5826.

Meistrich, M.L., and Drake, J.W. (1972) J. Mol. Biol. 66, 107-114.

Meuth, M., L'Heureux-Huard, N., and Trudel, M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 6505-6509.

Meuth, M. (1981) Mol. Cell. Biol. 1, 652-660.

Mosig, G. (1983a) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 362-374, American Society for Microbiology, Washington, D.C.

Mosig, G. (1983b) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 120-130, American Society for Microbiology, Washington, D.C.

Mufti, S. (1979) Virology 94, 1-9.

Muzyczka, N.R., Poland, R.L., and Bessman, M.J. (1972) J. Biol. Chem. 247, 7116-7122.

Neuhard, J. (1968) J. Bact. 96, 1519-1527.

Neuhard, J., and Munch-Petersen, A. (1966) Biochim. Biophys. Acta 114, 61-71.

Neuhard, J., and Thomassen, E. (1971) J. Bact. 105, 657-665.

Nilsson, S., Reichard, P., and Skoog, L. (1980) J. Biol. Chem. 255, 9552-9555.

North, T.W., and Mathews, C.K. (1977) Biochem. Biophys. Res. Comm. 77, 898-904.

North, T.W., Bestwick, R.K., and Mathews, C.K. (1980) J. Biol. Chem. 255, 6640-6645.

Nossal, N.G. and Alberts, B.M. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 71-81, American Society for Microbiology, Washington, D.C.

Nutter, L.M., and Cheng, Y.-C. (1984) Pharmac. Ther. 26, 191-207.

O'Donovan, G.A., and Neuhard, J. (1970) Bacteriol. Rev. 34, 278-343.

O'Donovan, G.A., Edlin, G., Fuchs, J.A., Neuhard, J., and Thomassen, E. (1971) J. Bact. 105, 666-672.

Owen, J.E., Schultz, D.W., Taylor, A. and Smith, G.R. (1983) J. Mol. Biol. 165, 229-248.

Pauling, C. (1968) Structural Chemistry and Molecular Biology (Rich, A., and Davidson, N., eds) pp. 383-398, W.H. Freeman & Co.

Payne, S.M., and Ames, B.N. (1982) Analyt. Biochem. 123, 151-161.

Peterson, G.L. (1983) Meth. Enz. 91, 95-119.

Petruska, J., and Goodman, M.F. (1985) J. Biol. Chem. 260, 7533-7539.

Pietrzykowska, I., Krych, M., and Shugar, D. (1983) Mutat. Res. 111, 119-133.

Pietrzykowska, I., Krych, M., and Shugar, D. (1985) Mutat. Res. 149, 287-296.

Pizer, L.I. and Cohen, S.S. (1962) J. Biol. Chem. 237, 1251-1259.

Platz, A. and Sjöberg, B.M. (1980) J. Bacteriol. 143, 561-568.

Pribnow, D., Sigurdson, D.C., Gold, L., Singer, B.S., Napoli, C., Brosius, J., Dull, T., and Noller, H.F. (1981) J. Mol. Biol. 149, 337-376.

Quiñones, A., and Piechocki, R. (1985) Molec. Gen. Genet. 201, 315-322.

Price, A.R. and Warner, H.R. (1969) Virology 37, 882-892.

Rabussay, D., and Geiduschek, E.P. (1977) Compr. Virol. 8, 1-196.

Rabussay, D. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 167-173, American Society for Microbiology, Washington, D.C.

Reha-Krantz, L.J. and Bessman, M.J. (1977) J. Mol. Biol. 116, 99-113.

Reha-Krantz, L.J. and Bessman, M.J. (1981) J. Mol. Biol. 145, 677-695.

Revel, H.R. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 156-165, American Society for Microbiology, Washington, D.C.

Ripley, L.S. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 4128-4132.

Ripley, L.S., and Shoemaker, N.B. (1983) Genetics 103, 353-366.

Ripley, L.S., Glickman, B.W., and Shoemaker, N.B. (1983) Molec. Gen. Genet. 189, 113-117.

Roguska, M.A., and Gudas, L.J. (1984) J. Biol. Chem. 259, 3782-3790.

Ronen, A., and Rahat, A. (1976) Mutat. Res. 34, 21-34.

Ronen, A., Rahat, A., and Halevy, C. (1976) Genetics 84, 423-436.

Ronen, A., Halevy, C., and Kass, N. (1978) Genetics 90, 647-657.

Ronen, A., and Halevy, C. (1980) Molec. Gen. Genet. 178, 397-401.

Rydberg, B. (1977) Biochem. Biophys. Acta 476, 32-37.

Salts, Y., and Ronen, A. (1971) Mutat. Res. 13, 109-113.

Scocca, J.J., Panny, S.R., and Bessman, M.J. (1969) J. Biol. Chem. 244, 3698-3706.

Shlomai, J. and Kornberg, A. (1978) J. Biol. Chem. 253, 3305-3312.

Siegel, E.C. and Vaccaro, K.K. (1978) Mutat. Res. 50, 9-17.

Singer, B.S. (1984) Molec. Gen. Genet. 193, 104-109.

Sinha, N.K., Morris, C.F., and Alberts, B.M. (1980) J. Biol. Chem. 255, 4290-4303.

Sinha, N.K., and Haimes, M.D. (1981) J. Biol. Chem. 256, 10671-10683.

Sinha, N.K., and Goodman, M.F. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 131-137, American Society for Microbiology, Washington, D.C.

Skopek, T.R., and Hutchinson, F. (1982) J. Mol. Biol. 159, 19-33.

Slabaugh, M.B., and Mathews, C.K. (1984) J. Virol. 52, 501-506.

Slabaugh, M.B., Johnson, T.L., and Mathews, C.K. (1984) J. Virol. 52, 507-514.

Smith, M.D., Green, R.R., Ripley, L.S., and Drake, J.W. (1973) Genetics 74, 393-403.

Snustad, D.P., Snyder, L., and Kutter, E. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 40-55, American Society for Microbiology, Washington, D.C.

Snyder, L. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 351-355, American Society for Microbiology, Washington, D.C.

Speyer, J.F. (1965) Biochem. Biophys. Commun. 1, 6-8.

Speyer, J.F., Karam, J.D., Lenny, A.B. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 693-697.

Streisinger, G., Okada, Y., Emrich, J., Newton, J., Tsugita, A., Terzaghi, E., and Inouye, M. (1966) Cold Spring Harbor Symp. Quant. Biol. 31, 77-84.

Sugino, A., and Drake, J.W. (1984) J. Mol. Biol. 176, 239-249.

Szyszko, J., Pietrzykowska, I., Twardowski, T., and Shugar, D. (1983) Mutat. Res. 108, 13-27.

Thelander, L. and Reichard, P. (1979) Ann. Rev. Biochem. 48, 133-158.

Topal, M.D. and Fresco, J.R. (1976) Nature (London) 263, 285-289.

Trudel, M., Van Genechten, T., and Meuth, M. (1984) J. Biol. Chem. 259, 2355-2359.

Ullman, B., Clift, S.M., Gudas, L.J., Levinson, B.B., Wormsted, M.A., and Martin, Jr., D.W. (1980) J. Biol. Chem. 255, 8308-8314.

Vaccaro, K.K. and Siegel, E.C. (1975) Molec. Gen. Genet. 141, 251-262.

Warner, H.R. and Barnes, J.E. (1966) Proc. Natl. Acad. Sci. U.S.A. 56, 1233-1240.

Warner, H.R. and Hobbs, M.D. (1967) Virology 33, 376-384.

Warner, H.R. and Duncan, B.K. (1978) Nature (London) 272, 32-34.

Warner, H.R., and Snustad, D.P. (1983) in Bacteriophage T4 (Mathews, C.K., Kutter, E.M., Mosig, G., and Berget, P.B., eds) pp. 103-109, American Society for Microbiology, Washington, D.C.

Watanabe, S.M., and Goodman, M.F. (1978) J. Virol. 25, 73-77.

Watson, J.D. and Crick, F.H.C. (1953a) Nature (London) 171, 737-738.

Watson, J.D. and Crick, F.H.C. (1953b) Nature (London) 171, 964-967.

Weinberg, G., Ullman, B., and Martin, Jr., D.W. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 2447-2451.

Weinstein, B.I., Bhardwaj, N., and Li, H. (1975) Analyt. Biochem. 68, 62-69.

Werner, R. (1968) Cold Spring Harbor Symp. Quant. Biol. 33, 501-508.

Weymouth, L.A., and Loeb, L.A. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1924-1928.

Wiberg, J.S. and Buchanan, J.M. (1964) Proc. Natl. Acad. Sci. U.S.A. 51, 421-428.

Wiberg, J.S. (1967) J. Biol. Chem. 242, 5824-5829.

Williams, W.E., and Drake, J.W. (1977) Genetics 86, 501-511.

Yarosh, D.B., Johns, V., Mufti, S., Bernstein, C., and Bernstein, H. (1980) Photochem. Photobiol. 31, 341-350.

Yeh, Y.C., Dubovi, E.J., and Tessman, I. (1969) Virology 37, 615-623.

Yeh, Y.C. and Tessman, I. (1972) Virology 47, 767-772.

Zaug, A.J., Kent, J.R., and Cech, T.R. (1984) Science 224, 574-578.

Zimmerman, S.B. and Kornberg, A. (1961) J. Biol. Chem. 236, 1480-1486.

APPENDICES

The dNTP Assay: Considerations and Calculations

The dNTP assay works by using dNTPs supplied by cell extracts and DNA polymerase, usually E. coli DNA polymerase I, for DNA synthesis on defined alternating copolymer templates (poly dAdT:poly dAdT or poly dIdC:poly dIdC). The assay can be used to measure dNTP concentrations in the range of 0.5 to 100 pmoles per reaction mixture, though the usual range is 2 to 40 pmoles per reaction mixture.

All four dNTPs are present in the cell extracts. By using the alternating copolymer templates, nucleic acid synthesis on the template is limited to using only two of the deoxyribonucleotides in the cell extracts as substrates for synthesis. In order to measure the concentration of a given dNTP in an extract, a large excess of radioactive (usually [^{3}H]dNTP) complementary dNTP is added to the reaction mixture. This leaves the unlabelled dNTP being measured, limiting in concentration for DNA synthesis. Measurement of unlabelled dNTP can be thought of in the following way. For every unlabelled dNTP (supplied by the cell extract) incorporated into the growning DNA strand, a labelled dNTP will subsequently be incorporated, until all of the limiting unlabelled dNTP has been used up. The amount of labelled dNTP incorporated will essentially be equal to the amount of unlabelled dNTP incorporated.

For example, to measure the dTTP concentration of a cell extract, an excess of [^{3}H]dATP is added to the reaction mixture, along with template (poly dAdT:poly dAdT), DNA polymerase, and an aliquot of cell extract. After DNA synthesis has been completed, the reaction mixtures are sampled for acid precipitable counts incorporated into the template. The amount of dTTP present in the cell extract and hence incorporated into the template is determined by comparing acid precipitable counts measured with cell extracts, to counts incorporated in reactions using known concentrations of dTTP. The dATP concentration of the cell extract would be measured in the same way, but by adding an

excess of [^{3}H]dTTP to the reactions instead of [^{3}H]dATP, and comparing the cell extract counts to dATP standards.

Since all four dNTPs are present in cell extracts, there will be some dilution in the specific activity of the added radioactive dNTP. To accurately calculate the true concentration of the dNTP being measured in the cell extract, this dilution of specific activity must be taken into account. Furthermore, to make the correction calculation, the uncorrected concentration of both the dNTP being measured and the complementary unlabelled dNTP in the extract must be measured. For example, before the correct dTTP concentration of a cell extract can be calculated, the dATP concentration must be determined. Likewise, before the correct dATP concentration in a cell extract can be calculated, the dTTP concentration must also be measured.

The correction factor is given by:

$$\text{correct } \mathbf{dNTP} \text{ concentration} = \mathbf{dNTP}^{*} \times \frac{\mathbf{complementary\ dNTP}^{*} + 100}{100}$$

where: **dNTP** is the true dNTP concentration

dNTP* is the uncorrected dNTP concentration measured in the cell extracts

complementary dNTP* is the uncorrected concentration of the complemenetary dNTP in the same cell extract as the **dNTP***.

dNTP Extraction Solutions and Recipies

60%Methanol/1%Toluene
50% (3.1 M)Trichloroacetic acid (TCA)
5% TCA
0.5 M Tri-n -octylamine/Freon
10 mM rXTP

50% (3.1 M)Trichloroacetic acid (TCA)

Add 500 ml water
to 500 g bottle of TCA (F.W. 163.4).
Adjust to 1 liter with water.
Store at 4 ^{0}C.

60%Methanol/1%Toluene

Mix 60 ml of methanol and 1.0 ml of toluene
with 39 ml water.

100 ml
Make fresh before use.
Keep on ice at 4 ^{0}C.

0.5 M Tri-n -octylamine/Freon
(TOA F.W.= 35 Density = 0.809 g/ml)

Add 2.19 ml of tri-n-octylamine (TOA)
to 7.81 ml of Freon.

10 ml
Make up fresh before use.
Keep on ice at 4 ^{0}C.

dNTP Extraction from T4 Infected E. coli

1) Dilute an overnight culture of cells 1:50 into fresh medium and grow with aeration to a density of 3×10^8 cells per ml (The cell titer can be determined by using a Petroff-Hauser counting chamber to count a sample of the cell culture with a microscope).

2) Immediately before infection, add L-tryptophan to 20 µg/ml and take a 1 ml sample of cells. Keep the cell sample on ice until the cells can be diluted and plated. To accurately determine the cell titer of the infections, plate 0.5 ml of a 10^{-6} dilution in duplicate.

3) Infect at a multiplicity of infection of 4 to 5 phage per cell, and allow the cultures to incubate 2 minutes without aeration (This allows the phage time to adsorb to the cells and inject their DNA.), then start aeration. (By convention, the start of phage infection [time = 0] begins after the 2 minute preincubation.)

4) Sample by rapid filtration (see notes below).

5) Immediately immerse the filters in cold (4 ^{0}C), 5% TCA and incubate for 30 minutes. Alternatively, extract in 60% methanol/1% toluene for 4 hours at 4 ^{0}C Add rXTP to about 10 µM (usually add 50 ul of 10 mM rXTP).

6) Pipet the extracts into cold 15 ml Corex centrifuge tubes and wash the filters with about 1/10 volume of cold 5% TCA or 60% methanol/1% toluene. Combine the wash with the extract and centrifuge at 10,000 x g. For TCA extracts, go to step 8. Lyophilize 60% methanol/1% toluene extracts.

7) Resuspend the residue in cold (4 ^{0}C), 5% TCA and centrifuge at 10,000 x g, 4 ^{0} C, for 30 minutes. (At this point, combine any samples that had to be split earlier in the extraction.)

8) Pipet off the supernatant and neutralize by extracting once with 1.5 volumes of cold 0.5 M tri-n-octylamine/Freon. (After adding the 0.5 M tri-n-octylamine/Freon, vortex each sample for 1 minute and then spin briefly to separate the phases.)

9) Check the pH of the upper (aqueous) phase with pH papers, which should be about pH 5.5 to 6.0, carefully recover and save the upper layer. (In order to avoid pipetting any of the lower phase, pipet 75% of the upper phase volume.) If the pH is less than pH 5.5, reextract <u>all</u> of the upper layer with another 1.5 volumes of 0.5 M tri-n-octylamine/Freon.

10) Lyophilize the saved upper layer and dissolve the residue in 0.25 ml of water or 1 X assay buffer. (This should make a clear, orange-yellow solution.)

11) Dilute the cell extracts and assay for dNTPs and rNTPs.

<u>Notes:</u>

1) How to use the Petroff-Hauser Cell Counting Slide.
   a) Make sure the slide and cover slip are clean and dry.
   b) Place the cover slip, flat (glass) side down, on top of the engraved squares on the slide base.
   c) Place a small drop of cell culture at the edge of the cover slip such that the liquid is drawn under the cover slip. If the space under the cover slip is over- or under-filled, the cells may stream through the microscope field, making counting difficult. Excess

liquid may be soaked up from the slide and cover slip with the corner of a dry Kimwipe.

d) Count cells using the microscope at 400 X magnification, with phase contrast. At 400 X magnification, once the slide is centered correctly, the microscope field will be filled with small squares. Count the cells in at least 10 of the small squares and multiply the average number of cells per single small square times 2×10^7 to get the titer of the cell culture in cells per ml.

2) The sample of uninfected cells may be kept on ice for 1 to 2 hours before plating. Usually plate on nutrient broth plates and incubate overnight at 37 ^{0}C.

3) When infecting the cells with phage, use a high titer phage lysate so that the volume of the cell culture is changed less than 5% after the phage have been added. Otherwise, the number of infected cells sampled will be different from the cell titer determined before infection.

4) How to Rapidly Filter Phage-Infected Cells.

a) For volumes of 25 ml to about 50 ml:

Rinse an empty 47mm filter holder with a few ml. of water, stack a 47mm Nucleopore Toyo GB 100R glass fiber prefilter on top of a 47mm Millipore AP 15 glass fiber prefilter and reassemble the filter holder. Use a small vacuum pump for sampling, with a 4 liter side arm flask to trap the filtrate. Immediatly after all the liquid has passed through the filters, immerse the filters in cold 60% methanol/1% toluene, or 5-10% TCA

b) For volumes of greater than 50 ml:

Same procedure as for smaller volumes (above) except use a 90mm filter holder and either a single 90mm 0.45µm. membrane filter (Millipore cat. no. HAWP 090 25, pore size 0.45 um.) or a combination of prefilter and membrane filter.

When using the vacuum pump to filter large volumes of aqueous solutions, contamination of the pump oil is a problem. This will cause the pump to work erratically and draw a weaker vacuum. It is very important to drain the pump oil every time after use to insure the pump continues to function reliably.

5) rXTP is added as a marker. This allows one to correct for losses of nucleotides during extraction and neutralization, by comparing the amount of rXTP in the neutralized cell extract to the amount of rXTP added to the 60% methanol/1% toluene or TCA.

dNTP Assay Stock Solutions

1.0 M Tris-OH
1.0 M Tris-Cl
1.0 M KH_2PO_4
1.0M K_2HPO_4
1.0 M $MgCl_2$
5.0 M NH_4OAc
100 mM dAMP
10 mg/ml BSA
0.1 unit/µl E. coli DNA polymerase I
0.63 mg/ml Template (alternating copolymers of poly dI-dC or poly dA-dT)
100 uM dNTP [one solution each of dATP, dGTP, dCTP (or HmdCTP), and dTTP]
dNTP standards [a set of dilutions for each dNTP, rangeing from 0.5 pmole/10 µl (0.05 µM) to 80 pmole/10 µl (8 µM)]
^{3}HdATP, ^{3}HdGTP, ^{3}HdCTP and ^{3}HdTTP (each at 1mCi/ml)
50% Trichloroacetic acid (TCA)
5% TCA/2% PPi
95% Ethanol
1 M Dithiothreitol
14.4 M Beta-Mercaptoethanol (β-ME)
Scintillation Cocktail

dNTP Assay Stock Solution Recipies

1.0 M Tris-OH

Dissolve
12.1 g Tris-OH (F.W. 121.1)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M Tris-Cl

Dissolve
15.8 g Tris-Cl (F.W. 157.6)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M KH_2PO_4

Dissolve
13.6 g KH_2PO_4 (F.W. 136.1)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M K_2HPO_4

Dissolve
22.8 gm K_2HPO_4-($3H_2O$) (F.W. 228.2)
in 80 ml water
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M $MgCl_2$

Dissolve
20.3 g $MgCl_2$-($6H_2O$) (F.W. 203.3)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

5.0 M NH_4OAc

Dissolve
38.5 g NH_4OAc (F.W. 77.1)
in 80 ml water.
Adjust to 100 ml with sterile water.
Do Not Autoclave
Store at room temperature.

100 mM dAMP

Dissolve
42 mg of dAMP-1.5 Na-$3H_2O$ (F.W. 420)
in 1 ml of water.
Store at -20 ^{0} C in lab freezer.

10 mg/ml BSA

Dissolve
10 mg of Bovine Serum Albumin (DNAse free)
in 1 ml water
or
make a dilution from BSA (DNAse free) supplied by a vendor like BRL, IBI or New England Biolabs.
Store at -20 ^{0}C in the lab freezer.

50% (3.1 M)Trichloroacetic acid (TCA)

Add
500 ml water
to 500 g bottle of TCA (F.W. 163.4).
Adjust to 1 liter with water.
Store at 4 °C.

5% TCA/2% PPi

Dissolve
2 g of sodium pyrophosphate
in 10 ml of 50% TCA
and 90 ml of water.
Make up fresh before using and keep at room temperature.

1.0 M Dithiothreitol (DTT)

Dissolve
1.55 g DTT
in 10 ml of 0.01 M NaOAc pH 5.2.
Do Not Autoclave
Dispense 1 ml aliquots into screwcapped 1.5 ml. microfuge tubes.
Store at -20 °C in the walk-in freezer.

dNTP Assay Buffers And Solutions

1.5 X dNTP Assay Buffer

75 mM Tris pH 8.3	4.5 ml of 1 M Tris-OH 3.0 ml of 1 M Tris-Cl
7.5 mM $MgCl_2$	0.75 ml of 1 M $MgCl_2$
	92.0 ml of water
	100 ml

Check the pH.
Autoclave.
Store at room temperature.

Immediately before use, add β-mercaptoethanol to a final concentration of 1.5 mM. (Add 1 µl of a 1.44 M solution of β-ME per ml of 1.5 X dNTP assay buffer.)
Keep on ice until used.

200 mM Phosphate Buffer pH 7.0

200 mM KPO_4; pH 7.0	3.9 ml of 1 M KH_2PO_4 (monobasic)
	6.1 ml of 1 M K_2HPO_4 (dibasic)
	40.0 ml of water
	50 ml

Check the pH.
Autoclave.
Store at room temperature.

DNA Polymerase Dilution Buffer

100 mM KPO_4 pH 7.0	5 ml of 200 mM KPO_4 buffer pH 7.0
1 mM DTT	10 µl of 1 M DTT
50% Glycerol	5 ml of sterile glycerol
	10 ml

Aliquot 1 ml fractions into sterile microfuge tubes.
Store at -20 °C in the walk-in freezer.

0.1unit/µl E. coli DNA Polymerase

Make a stock solution of E. coli DNA polymerase I at 0.1unit/µl, with E. coli DNA polymerase I dilution buffer, from the E. coli DNA polymerase I solution supplied by the vendor.

This solution will be good for a couple of months if stored at-20 °C.
Both the 0.1unit/µl and the undiluted E. coli DNA polymerase I must be kept in the walk-in, -20 °C, cold room or a freezer that is not a "Frost Free" freezer. Frost Free freezers regularly warm up to defrost and this temperature change will cause the DNA polymerase to go bad.

dNTP Solutions

Stock solution concentrations of each of the dNTPs are standardized spectrophotometrically, make up as follows:

Dissolve
about 6 mg of dNTP
in 0.5 ml of water.

Determine the dNTP concentration using extinction coefficients given below.
Adjust the dNTP concentration to 10 mM with water.

	pH 2.0			pH 7.0		
Nucleotide	λ max nm		ε $M^{-1}cm^{-1}$	λ max nm		ε $M^{-1}cm^{-1}$
dATP	258		14,300	259		15,300
dGTP	255	(pH 1)	11,800	253		13,700
dCTP	280		13,500	271		9,300
hmdCTP	284	(pH 1)	12,500	275		7,700
dTTP	267		10,200	267		10,200
dUTP	260		9,800	260		9,800
BrdUTP	280		9,900	----		------
rXTP	263	(pH 3)	8,950	248	(pH 8)	10,200

Make a 1mM working stock solution, from the spectrophotometrically standardized 10 mM dNTP solutions, of each dNTP that you will be assaying (for most assays, make stock solutions of dATP, dGTP, dCTP and dTTP).

The following dNTP dilutions are made from the 1mM stock solution (at least 1 ml of each):

dNTP	Concentration
ALL	100 µM
dATP, dTTP and dCTP	8 µM (80 pmol/10 µl), 4 µM (40 pmol/10 µl), 2 µM (20 pmol/10 µl), 1µM (10 pmol/ 10 µl), 0.5 µM (5 pmol/ 10 µl).
dGTP	4 µM (40 pmol/10 µl), 2 µM (20 pmol/10 µl), 1µM (10 pmol/10 µl), 0.5 µM (5 pmol/10 µl), 0.2 µM (2 pmol/10 µl), 0.1 µM (1 pmol/10 µl), 0.05 µM (0.5 pmol/10 µl).

TEMPLATE

poly dA-dT (0.75 mg/ml; ca. 2.0 mM)
poly dI-dC (0.75 mg/ml; ca. 2.0 mM)

Dissolve template in the bottle, with water, to give an approximate concentration of 1 mg/ml. Determine the template concentration using the specifications supplied by the vendor or by using the following values:

$Abs._{260}$ Units/mg dA-dT, pH 7.0 = 18.0
$Abs._{260}$ Units/mg dI-dC, pH 7.0 = 12.5
or
poly dA-dT; pH 7.0, @260 nm: $\epsilon_{M^{-1}cm^{-1}} = 6700$
poly dI-dC; pH 7.0, @251 nm: $\epsilon_{M^{-1}cm^{-1}} = 6900$
(Dilute the template into 0.02 M phosphate buffer, 0.1 M NaCl, pH 7.0 to take the

absorbance.)

Adjust the template concentration, with water, to 0.75 mg/ml.

Aliquot 1 ml fractions into sterile microfuge tubes.

Store at -20 ^{0}C.

Scintillation Cocktail

Add

12 g of PPO (2,5-Diphenyloxazole)
and 0.4 g of Dimethyl POPOP
(1,4-bis-[4-Methyl-
5-phenyl-2-oxazolyl]benzene)

to 4 liters of scintillation grade toluene.

Stir until dissolved.

Store at room temperature in a tightly capped container.

dNTP Assay

1) Dilute cell extracts with water or 1X dNTP assay buffer so the concentration of each dNTP being assayed is in the range of 0.5 pmol/10 µl (0.05 µM) to 40 pmol/10 µl (4 µM). Reasonable dilutions of cell extracts to use for dNTP assays are: dGTP, 2-fold and 4-fold; dATP, dCTP and dTTP, 4-fold and 8-fold.

2) Label filters, in pencil, and saturate each filter with 100 µl of 5% TCA/2% PPi. Let air dry before sampling.

3) Add 10 µl of diluted cell extract to the sample reaction tubes and 10 µl of dNTP standard to the standard curve reaction tubes (use 500 µl microfuge tubes for reaction tubes). If cell extracts are to be treated with T4-dCTPase/dUTPase or E. coli-dUTPase, see dNTP Assay Notes below. Keep cell extracts on ice until addition of reaction mix.

   Use the following concentrations of dNTP standards to construct standard curves:

   dGTP: 0 pmol/10 µl (water), 2 pmol/10 µl (0.2 µM), 5 pmol/10 µl (0.5 µM), 10 pmol/10 µl (1 µM) and 20 pmol/10 µl (2 µM).

   dATP, dCTP and dTTP: 0 pmol/10 µl (water), 5 pmol/10 µl (0.5 µM), 10 pmol/10 µl (1 µM), 20 pmol/10 µl (2 µM) and 40 pmol/10 µl (4 µM).

4) Make the dNTP assay reaction mix. Each reaction tube will contain in a final volume of 100 µl:

45 mM Tris, pH 8.3
4.5 mM $MgCl_2$
1 mM β-mercaptoethanol
1 µM [^{3}H]dNTP complementary to the dNTP being assayed (ca. 10 mCi/µm)
1 unit/ml *E. coli* DNA polymerase I
ca. 8 µg/ml (20 µM) template
0.2 mg/ml BSA
10 mM dAMP
10 µl of dNTP standard or diluted cell extract

---

100 µl

Each reaction tube will thus contain the following:

	dATP, dGTP, and dTTP Reaction Tubes	dCTP Reaction Tube
1.5 X dNTP Assay Buffer (1.5 mM β-mercaptoethanol)	60 µl	60 µl
BSA (10 mg/ml)	2 µl	2 µl
Template (0.75 mg/ml)	1 µl	poly dI-dC 1 µl
dNTP (100 µM)	1 µl	dGTP 1 µl
^{3}H dNTP (1 mCi/ml)	1 µl	[^{3}H]dGTP 2-3 µl
E. coli DNA polymerase I (0.1 unit/µl)	1 µl	1 µl
dAMP (100 mM)	10 µl	10 µl
water	14 µl	13-12 µl
	90 µl	90 µl
dNTP standard or diluted cell extract	10 µl	10 µl
	100 µl	100 µl

The dNTP reaction mix consists of all the above components except the dNTP standard or diluted cell extract. To make the reaction mix, multiply the volume of each component given above, by the number of reactions or assays to be done [this will include the 5 reactions per dNTP needed to construct the standard curves and however many dilutions of cell extracts being assayed for dNTPs; it is a good idea to add extra (5% to 10% more) reactions to insure there will be enough reaction mix to aliquot out].

Add all the components together except for template and E. coli DNA polymerase I.

Keep on ice.

Add the template and E. coli DNA polymerase I last, immediately before starting the reactions.

5) Start the dNTP assay reactions by adding 90 µl of dNTP reaction mix to the reaction tubes containing the dNTP standards or diluted cell extracts.

6) Incubate the reactions at 37 ^{0}C in a water bath and stop the reactions by pipeting 25 µl samples, at 60, 75 and 90 minutes, onto dried 5% TCA/2% PPi-presoaked filters.

7) Wash the samples in 5% TCA/2% PPi. This may be done using either of two methods.

(a) If 2.4 cm glass fiber or paper filter discs are used, batch wash the discs three times in cold 5% TCA/2% PPi, 15 minutes per wash, with about 2 ml. of 5% TCA/2% PPi per filter disc. (One may wash filter discs from different dNTP assays without cross-contaminating the filter discs.)

Batch wash the filter discs twice, 15 minutes per wash, in cold, 95% ethanol (2 ml of 95% ethanol per disc).

Let the discs air dry overnight or dry under a heat lamp for about 1 hour, place the discs in scintillation vials containing 5 ml of scintillation cocktail and count for 5 minutes (5% error) using the [^{3}H] window.

(b) If Whatman 3 Chr or 3mm Chr paper filter sheets are used for sampling, wash the sheets at room temperature by ascending chromatography with 5% TCA/2% PPi as the solvent.

Allow the solvent front to run within about 1 cm of the end of the sheet. (See <u>dNTP Assay Notes</u> below.)

Cut or tear the strips of sample squares off the bottom of the sheets and batch wash twice in 95% ethanol, 15 minutes per wash, 20 ml of 95% ethanol per strip.

Let the strips air dry overnight or dry under a heat lamp for

about 1 hour, cut the strips into individual sample squares, place the sample squares in scintillation vials containing 5 ml of scintillation cocktail and count for 5 minutes (5% error) using the [^{3}H] window.

dNTP Assay Notes:

1) How to use Whatman 3 mm Chr or Whatman 3 Chr Filter Sheets for Sampling Acid Precipitable Counts.

The advantages to this method are that there is less liquid [^{3}H]-TCA wastes generated and the washes take a little less time. The disadvantages are it takes more time to set up and label the filter sheets than the filter discs and one must cut out the sample squares after the washes are finished and the filter sheets are dried.

a) Cut out filter sheets 20 cm wide by 19 to 20 cm high from 46 cm X 57 cm Whatman 3 mm Chr or Whatman 3 Chr filter paper. Draw , in pencil, a row of 10 squares, each square 2 cm to a side with the bottom of the row 2 cm from the bottom edge of the filter paper. Label or number each square, in pencil, along one of the inside edges of the square.

b) Before using the sheets for sampling, add 0.1 ml of 5% TCA/2% PPi to each square and allow to dry.

c) Sample directly onto the dry 5% TCA/2% PPi-presoaked squares.

d) To wash the samples, fill the glass dish in the chromatography tank with fresh 5% TCA/2% PPi and suspend the filter sheets such that the bottom 1 cm of each sheet is in the 5% TCA/2% PPi solution. The sheets

are held in place by clamping the top of each sheet to a horizontal glass rod above the 5% TCA/2% PPi reservoir. Replace the top of the chromatography tank to prevent evaporation of the 5% TCA/2% PPi solution. It usually takes about 90 min for the 5% TCA/2% PPi to ascend to the top of the sheets. With care, it is possible to wash about 8-to 9-sheets at a time.

e) Cut or tear the row of sample squares off from the remainder of the filter sheet, wash twice in 95% ethanol (about 20 ml of ethanol per row of squares per wash) and air dry overnight or dry for about 1 hour under a heat lamp. One may batch wash the rows of filter sheets in 95% ethanol without cross-contamination of sample squares.

f) Cut out the sample squares, place each square in a vial containing 5 ml of scintillation cocktail and count for 5 min (5%) error.

2) Treatment of Cell Extracts with T4- dCTPase/dUTPase or E. coli- dUTPase.

a) Dilute the T4- dCTPase/dUTPase or E. coli- dUTPase 10-fold into 1.5 X dNTP assay buffer (1.5 mM β-mercaptoethanol) to give a concentration of 1 X 10^{-4} units/µl [1 unit of enzyme will catalyze the cleavage of 1 µmole dUTP (or dCTP) to dUMP (or dCMP) per minute at 37 ^{o}C].

b) Add 10 µl of cell extract to reaction tubes. [It is a good idea to set up duplicate tubes, one tube will get enzyme and the other will be a control that will get an equal volume of 1.5 X dNTP assay buffer (1.5 mM β-mercaptoethanol).]
Keep tubes on ice until addition of enzyme.
Set up the standard curve tubes the same as the control tubes [10 µl of dNTP and 10 ul of 1.5 X dNTP assay buffer (1.5 mM β-mercaptoethanol)] but do not incubate at 37 ^{o}C for 30 minutes.

c) Add 10 µl of diluted enzyme to the treatment tube and 10 µl of 1.5 X dNTP assay buffer (1.5 mM β-mercaptoethanol) to the control tube.
Incubate for 30 minutes at 37 ^{o}C.

d) Chill the reaction tubes on ice a few minutes and spin briefly to remove condensation off the walls of the reaction tubes.

e) Start the dNTP assay by adding 80 µl of dNTP reaction mix made from the recipe below (adjusted to add 10 ul of water less per reaction tube) and proceed from step 6 of the dNTP Assay.

	dATP, dGTP, and dTTP Reaction Tubes	dCTP Reaction Tube
1.5 X dNTP Assay Buffer (1.5 mM β-mercaptoethanol)	60 µl	60 µl
BSA (10 mg/ml)	2 µl	2 µl
Template (0.75 mg/ml)	1 µl	poly dI-dC 1 µl
dNTP (100 µM)	1 µl	dGTP 1 µl
[^{3}H] dNTP (1 mCi/ml)	1 µl	[^{3}H]dGTP 2-3 µl
E. coli DNA polymerase I (0.1 unit/µl)	1 µl	1 µl
dAMP (100 mM)	10 µl	10 µl
water	4 µl	3-2 µl
	80 µl	80 µl

3) Problems Encountered.

While the dNTP assay is a fairly reliable and easy assay to do, it is also easy to screw-up. Fortunately, commonly encountered problems are easy to identify and easy to fix. The following, lists some of the problems Janet Leeds, I (Geoff Sargent) and other labs have had to deal with. I have tried to organized the list to reflect the common sources of trouble in the assay since we have incorporated in the assay protocol some 'preventive' solutions to the problems.

The references in the bibliography are an excellent source for troubleshooting as well.

a) DNA Polymerase Problems

DNA polymerase is probably the most frequent cause of problems with the dNTP assay. It is important to use the best quality polymerase availible to insure accurate results and keep the assay running reliably. We have used polymerase supplied by Worthington and received only one bad batch in 4 years.

Reichard's lab recommends the "endonuclease-free" polymerase from Boehringer-Mannheim.

Old batches of DNA polymerase, especially those kept in "frost-free" freezers, seem to suffer a greater loss of polymerase activity relative to exonuclease activity. This will show up most dramatically in the standard curves, where over the time course of sampling, for a given concentration of dNTP, the counts will fluctuate. For example, if a 20 pmol TTP standard had 20,000 counts per minute when sampled at 60 min., 75 min. and 90 min. with "good" polymerase; "bad" polymerase might give the following results:

60 minutes, 17,000 cpm; 75 minutes, 19,000 cpm; 90 minutes, 14,000 cpm.

The solution is to keep the enzyme in a non"frost-free" freezer; do not dilute or otherwise manipulate the polymerase out of the freezer (even keeping the polymerase on ice for short periods of time will speed-up the enzyme going bad); and finally, do not be surprised if the enzyme goes bad after 6 months to a year in storage.

In the past, in our hands, commercial preparations of Klenow fragment of E. coli DNA polymerase I (lacking the 3' to 5' exonuclease activity of the polymerase) have not given good results with the dNTP assay. There are now suppliers that sell cloned sources of Klenow, so it might be worth another try.

The T4 DNA polymerase 3' to 5' exonuclease is 10-to 100-times more active than E. coli DNA polymerase I exonuclease and would probably not work very well with the dNTP assay.

High concentrations of deoxyribonucleoside monophosphate inhibit 3' to 5' exonuclease activity. dAMP is added is added to the reaction mix to inhibit the exonuclease activity and extend the time over which the reactions can be sampled. This might also allow one to use polymerase whose quality is marginal or polymerase that is beginning to go bad.

Salts present in the cell extracts can affect polymerase activity. This will prevent maximal incorporation of dNTPs into the synthesizing strand, giving fewer counts than expected in the samples. For example, salt inhibition of DNA polymerase could be the culprit for two dilutions of the same cell extract where the less dilute sample has fewer counts incorporated than the more dilute sample. The solution is to use at least a two-fold dilution of cell extracts in the dNTP assay, regardless of the extraction and neutralization procedure used.

b) Template Problems

The quality of template from the supplier is usually good enough for most dNTP assays. However, there may be deoxyribonucleotides present in the template preparations left over from the synthesis of template, that will cause high background counts. Additionally, high background counts seem to be worse with poly dI-dC. Ideally, in the standard curves with no dNTP added, there should be less than 100 cpm incorporated in the samples; while in practice, 1000 cpm may be incorporated in the standard that has no dNTP. Unless one is trying to measure very small quantities of dNTPs (less than 1 pmol) or doing double label experiments, high background counts can be tolerated.

We purchase template from Pharmicia.

Two methods that can be used to clean up template preps are:

1) Precipitate the template.
   - Add 1/10 volume of 5 M NH_4OAc to the template solution.
   - Add 2.5 volume of -20 ^{0}C, 95% EtOH.
   - Incubate for 2 hours at -20 ^{0}C.
   - Spin in the microfuge for 30 minutes and discard the supernatant.
   - Wash the pellet once with -20 ^{0}C, 70% EtOH.
   - Spin in the microfuge for 30 minutes and discard the supernatant.
   - Dry the pellet in the SpeedVac or lyophilizer.
   - Redissolve the template to 0.75 mg/ml.

Do not be tempted to precipitate the template at -80 ^{0}C as that might bring down salts as well as free deoxyribonucleotides.

2) Dialyze the template.

Dialyze: 24 hours against 1000 volumes of 1M NaCl/ 1 mM Tris, pH 7.4/1 mM EDTA, 4 ^{0}C.

2 X 6 hours against 1000 volumes of 10 mM NaCl/1 mM Tris, pH 7.4/1 mM EDTA, 4 ^{0} C.

If the template needs to be concentrated after dialysis, precipitate it following the directions above.

c) Other Quirks

When the dNTP assay is working well, the standard curves should have a slope of 1000 cpm incorporated per pmol of dNTP. However, dCTP assays rarely will achieve this effeciency of incorporation. The separate assay recipes (given above) for dCTP assays will improve the assay somewhat; but it is interesting to note that increasing the specific activity of [^{3}H]dGTP two-fold does not increase the counts incorporated two-fold. Since the dCTP assay is also the most suseptible to high background counts, there may be a greater contamination of deoxynucleotides in the poly dI-dC template preparation than the poly dA-dT. Therefore, the greatest improvement gained in precipitating or dialyzing template preparations might be in the dNTP assay for dCTP.

dNTP Extraction, Neutralization AND Assay References

Chen, S.-C., Brown, P. R., and Rosie, D. M. (1977) Extraction Procedures for Use Prior to HPLC Nucleotide Analysis Using Microparticle Chemically Bonded Packings *J. Chromatographic Science* **15,** 218-221

Hunting, D. and Henderson, J. F. (1982) Methods For The Determination Of Deoxyribonucleoside Triphosphate Concentrations *Methods in Cancer Research* **20,** 245-284

Hunting, D. and Henderson, J. F. (1981) Determination of Deoxyribonucleoside Triphosphates using DNA Polymerase: A Critical Evaluation. *Can. J. Biochem.* **59,** 723-727

Khym, J. X. (1975) An Analytical System for Rapid Separation of Tissue Nucleotides at Low Pressures on Conventional Anion Exchangers. *Clinical Chemistry* **21,** 1245-1252

Mathews, C. K. (1975) Giant Pools of DNA Precursors in Sea Urchin Eggs. *Exptl. Cell Res* **92,** 47-56

Nicander, B., and Eriksson, S. (1985) *EMBO Practical Course on DNA Precursor Metabolism.* Experimental Manual.

North, T. W., Bestwick, R. K., and Mathews C. K. (1980) Detection of Activities that Interfere with the Enzymatic Assay of Deoxyribonucleoside 5'-Triphosphates *J. Biol. Chem* **255,** 6640-6645

Payne, S. M. and Ames, B. N. (1982) A Procedure for Rapid Extraction and High-Pressure Liquid Chromatographic Separation of the Nucleotides and Other Small Molecules from Bacterial Cells *Analytical Biochemistry* **123,** 151-161

Shlomai, J. and Kornberg, A. (1978) Deoxyuridine Triphosphatase of Escherichia coli. Purification, Properties, and Use as a Reagent to Reduce Uracil Incorporation Into DNA *J. Biol. Chem.* **253,** 3305-3312

Skoog, L. (1970) An Enzymatic Method for the Determination of dCTP and dGTP in Picomole Amounts *Eu. J. Biochem.* **17,** 202-208

RNA Sequencing

RNA-Work Stock Solutions

1.0 M Tris-OH	100 ml	
1.0 M Tris-Cl	100 ml	
1.0 M NaCl	100 ml	
1.0 M $MgCl_2$	100 ml	
1.0 M MgOAc	100 ml	
5.0 M NH_4OAc	100 ml	
0.5 M EDTA	100 ml	
1.0 M NaOAc pH 5.2	100 ml	
0.2 M NaOAc pH 5.2		
Saline		
H_2O	2 x 100 ml	
1.0 M Dithiothreitol(DTT)	10-20 ml	
0.1 M Spermidine-Cl_3	10 ml	
1 mM EDTA pH 8.0		10 ml
50% Phenol/48% $CHCl_3$/2% isoamyl alcohol		500 ml
water saturated Ethyl Ether		
95% EtOH		
70% EtOH		

Notes:

1) All the solutions used from the phenol extraction on, when extracting RNA from cells, should be made as RNAse-free as is possible. That means wearing gloves, in order to minimize contaminating with finger RNAses when handling glassware and plasticware that contain these solutions.
2) Autoclaving does not kill all RNAses. Glassware and spatulas used for RNA-work should be baked at 400-450 ^0F, 4 hours, before using to store or make these solutions. Assume packaged plasticware is essentially RNAse free as long as only you have opened or used the plasticware and

are certain it has not been contaminated with RNAses.

3) To minimize contamination of dry reagents with RNAses by other people in the lab, buy reagents especially for RNA work and label accordingly. Wear gloves when handling these bottles and use baked spatulas to remove reagents out of the bottle. If an excess of reagent is taken out, throw it away rather than putting it back in the bottle. If you suspect that a reagent has been contaminated throw it out and order fresh reagent.

4) Water used to make all the solutions is house distilled water that has either been deionized and glass-distilled or comes from a Millipore *milli-q* Reagent Water System (the usual source, located in the wash-room). Water is autoclaved before use, and solutions, once made, are autoclaved.

RNA-Work Stock Solution Recipies

1.0 M Tris-OH

Dissolve
12.1 g Tris-OH (F.W. 121.1)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M Tris-Cl

Dissolve
15.8 g Tris-Cl (F.W. 157.6)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M NaCl

Dissolve
5.8 g NaCl (F.W. 58.44)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M $MgCl_2$

Dissolve
20.3 g $MgCl_2$-($6H_2O$) (F.W. 203.3)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

1.0 M MgOAc

Dissolve
21.45 g MgOAc-($4H_2O$) (F.W. 214.5)
in 80 ml water.
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

5.0 M NH_4OAc

Dissolve
38.5 g NH_4OAc (F.W. 77.1)
in 80 ml water.
Adjust to 100 ml with water.
Do Not Autoclave
Store at room temperature.

0.5 M EDTA pH 8.0

Dissolve
18.6 g EDTA-Na_2-($2H_2O$) (F.W. 372.2)
in 80 ml water.
Adjust to pH 8.0 with ca. 2.0 g NaOH pellets
(check with pH papers).
Adjust to 100 ml with water.
Autoclave. Store at room temperature.

1.0 M NaOAc pH 5.2

Dissolve
13.6 g NaOAc-($3H_2O$) (F.W. 82.03)
in 80 ml water.
Adjust to pH 5.2 with 1.9 ml of glacial acetic acid (check with pH papers).
Adjust to 100 ml with water.
Autoclave.
Store at room temperature.

0.2 M NaOAc pH 5.2

Dilute 1.0 M NaOAc pH 5.2, 1:5 with water
Autoclave.
Store at room temperature.

Saline
0.85% (w/v) NaCl

Dissolve
8.5 g NaCl
Adjust to 1 liter with water
Autoclave.
Store at room temperature.

1.0 M Dithiothreitol (DTT)

Dissolve
1.55 g DTT (F.W. 154.3)
in 10 ml of 0.01 M NaOAc (pH 5.2.).
Do Not Autoclave
Dispense 1 ml aliquots into screwcapped 1.5 ml microfuge tubes.
Store at -20 °C in the walk-in freezer.

0.1 M Spermidine

Dissolve
0.26 g spermidine-Cl_3 (F.W. 254.6)
in 10 ml water.
Do Not Autoclave
Dispense 1 ml aliquots into screwcapped 1.5 ml microfuge tubes.
Store at -20 °C in the walk-in freezer.

1 mM EDTA pH 8.0

Add
0.02 ml of 0.5 M EDTA pH 8.0
to 10 ml of water.
Store at room temperature.

50% Phenol/48% $CHCl_3$/2% isoamyl alcohol

Melt 100 ml of redistilled phenol at 65 °C
Dissolve 0.1 gm 8-hydroxyquinoline to the melted phenol.
Extract 3 times with 0.2 M NaOAc pH 5.2 (check the pH of the aqueous phase with pH papers; should be pH 5.2 when done)
Add 96 ml of $CHCl_3$.
Add 4 ml of isoamyl alcohol.
Store under 0.2 M NaOAc pH 5.2 at 4 °C.

Water saturated ethyl ether

Add an equal volume of water to anhydrous ethyl ether.
Shake well.
Store in a tightly capped bottle, at room temperature, in a hood.

95% EtOH

Store opened bottles at -20 °C in a freezer.

70% EtOH

Add 74 ml of 95% EtOH
to 26 ml of water.
Store opened bottles at -20 °C in a freezer.

Sequencing and End Labelling Buffers

5X Annealing Buffer (RNAse Free)

250 mM Tris pH 8.9	0.3 ml of 1.0 M Tris-Cl
(pH 8.3-8.6 at 48 ^{0}C)	2.2 ml of 1.0 M Tris-OH
300 mM NaCl	3.0 ml of 1.0 M NaCl
50 mM DTT	0.5 ml of 1.0 M DTT
	2.5 ml of water
	10.0 ml

Check the pH with pH papers.
Dispense 1.0 ml aliquots into 1.5 ml screwcapped microfuge tubes.
Store at -20 ^{0}C in the walk-in freezer.

5 X Reverse Transcriptase Buffer (RNAse Free)

250 mM Tris pH 8.9	0.3 ml of 1.0 M Tris-Cl
(pH 8.3-8.6 at 48 ^{0}C)	2.2 ml of 1.0 M Tris-OH
300 mM NaCl	3.0 ml of 1.0 M NaCl
50 mM DTT	0.5 ml of 1.0 M DTT
150 mM MgOAc	1.5 ml of 1.0 M MgOAc
	1.0 ml of water
	10.0 ml

Check the pH with pH papers.
Dispense 1.0 ml aliquots into 1.5 ml screwcapped microfuge tubes.
Store at -20 ^{0}C in the walk-in freezer.

10X Young's Lysing Buffer (RNAse Free)

0.5 M Tris pH 6.8	4.83 ml of 1.0 M Tris-Cl
	0.17 ml of 1.0 M Tris-OH
20 mM EDTA	0.4 ml of 0.5 M EDTA pH 8.0
10% SDS	1.0 g of sodium dodecyl sulfate
	4.6 ml water
	10.0 ml

Check the pH with pH papers.
Dispense 1.0 ml aliquots into 1.5 ml screwcapped microfuge tubes.
Store at room temperature.

10X Kinase Buffer I (RNAse Free) (see: Maniatis p.122, Forward Reaction-Using DNA molecules with protruding 5' termini as template)

0.5 M Tris pH 7.6	3.85 ml of 1.0 M Tris-Cl
	1.15 ml of 1.0 M Tris-OH
0.1 M $MgCl_2$	1.0 ml of 1.0 M $MgCl_2$
50 mM DTT	0.5 ml of 1.0 M DTT
1 mM Spermidine	0.1 ml of 0.1 M Spermidine
1 mM EDTA	0.02 ml of 0.5 M EDTA pH 8.0
	3.3 ml of water
	10.0 ml

Check the pH with pH papers.
Dispense 1.0 ml aliquots into 1.5 ml screwcapped microfuge tubes.
Store at -20 ^{0}C in the walk-in freezer.

10X Polynucleotide Kinase (PNK) Dilution Buffer

0.5 M Tris pH 7.6	3.85 ml of 1.0 M Tris-Cl
	1.15 ml of 1.0 M Tris-OH
10 mM DTT	0.1 ml of 1.0 M DTT
1 mM EDTA	0.02 ml of 0.5 M EDTA pH 8.0
	4.88 ml of water
	10 ml

Aliquot 1 ml fractions into screwcapped microfuge tubes.

To make 1.5X dilution buffer:

0.15 ml 10X PNK dilution buffer
0.75 ml glycerol
0.10 ml water

1.0 ml

To make a working stock of PNK at 10 units/µl, add 5 µl of U.S. Biochemical PNK (30 units/µl) to 10 µl 1.5X PNK dilution buffer.

Store at -20 ^{0}C in the walk-in freezer.

5X dNTP Mix (RNAse Free)

A mix of all 4 deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP), each at 2.0 mM final concentration in 1X annealing buffer.

Dispense 1.0 ml aliquots into 1.5 ml screwcapped microfuge tubes.

Store at -20 ^{0}C in the walk-in freezer.

5X ddNTP Mix (RNAse Free)

4 different dideoxyribonucleoside triphosphate solutions (ddATP, ddCTP, ddGTP and ddTTP), each at 250 µM in 1X annealing buffer.

Dispense 1.0 ml aliquots into 1.5 ml screwcapped microfuge tubes.
Store at -20 ^{0}C in the walk-in freezer.

Note: The concentrations of these solutions need to be very accurate for the sequencing reactions. One should make a concentrated (ca. 10 mM) stock solution of the nucleotides whose concentration is determined spectrophotometrically, using the table of extinction coefficents below. Use the stock solutions to make dilutions of the nucleotides for the working solutions above.

Nucleotide	pH 2.0 λ max nm		pH 2.0 $\epsilon_{M^{-1}cm^{-1}}$	pH 7.0 λ max nm	pH 7.0 $\epsilon_{M^{-1}cm^{-1}}$
A	258		14,300	259	15,300
G	255	(pH 1.0)	11,800	253	13,700
C	280		13,500	271	9,300
T	267		10,200	267	10,200
U	260		9,800	260	9,800

Preparation of RNA from T4 Infections

Notes:

1) It may be necessary to adjust the post-infection sampling time in order to sample when a particular species of RNA is most abundant.
2) Multiplicity of infections (moi) are about 10 phage per cell.
3) Starting with the phenol extraction, use reagents and glassware/plasticware reserved for RNA-Work Only.

Procedure:

1) Dilute an *E. coli* B overnight, grown in M9 at 30 ^{0}C, 1:50 into M9 and grow with bubbling (or shaking) at 30 ^{0} C to a titer of 3×10^{8} cells per ml (This generally takes at least 4 hrs, probably closer to 6 hrs)

2) Immediately before infection, add L-tryptophan to 20 µg/ml.

3) Aliquot 10 ml of cells into bubbling tubes (or shaking flasks or tubes), infect with phage (moi=10) and aerate vigorously at 30^{0} C.

4) At 12 min post-infection, pour infected cells into sterile 15 ml Corex centrifuge tubes and chill to 4 ^{0}C in an ice-water bath.

5) Spin down chilled, infected cells at 10,000 x g, 4 ^{0}C for 15 min, discard the supernatant and take off as much residual liquid remaining in the tube as possible.

6) Resuspend the infected cell pellet with 225 µl of cold saline and transfer to cold 1.5 ml microfuge tubes.
(An indication that there was a good infection is the pellet will be next to impossible to resuspend. It will pipet as

a gooey mess.)

7) Add 25 µl 10X Young's lysing buffer to each tube and incubate the tubes at 68 ^{0}C for 3.0 min (The solutions should be clear and very viscous after this step).

8) Immediately add 50 µl of 1.0 M NaOAc pH 5.2 to the lysed cells and mix as well as possible.

9) Extract the lysed cells with an equal volume of Phenol (50% phenol/48% $CHCl_3$/2% isoamyl alchol, equilibrated with 0.2 M NaOAc pH 5.2), mix well and spin in microfuge for 10 min (Usually 2-3 extractions are all that is necessary. When recovering the aqueous phase, in the initial extractions, it is ok to get some of the interface. When recovering the aqueous phase in the last phenol extraction, avoid pipeting any phenol or any of the trash at the interface).

10) Add 1/10 volume of 5 M NH_4OAc, 2.5 volumes of cold (-20 ^{0}C) EtOH to each tube, and incubate at least 2 hrs at -20 ^{0}C. Spin down the nucleic acids for 30-40 min at 4 ^{0}C in the microfuge.

11) Discard the supernatant, wash the pellets once with cold (-20 ^{0}C) 70% EtOH and spin for 5 min at 4 ^{0}C in the microfuge.

12) Discard the supernatant, dry the pellet in the SpeedVac and resuspend the dried pellet in 30 µl of 1 mM EDTA. Store the resuspended nucleic acids at -20 ^{0}C (The $Abs._{260}$ should be about 100 and the $Abs._{260/280}$ should be close to 2.)

Dideoxysequencing OF T4 mRNA

[Adapted by Geoff from procedure learned in L. Gold's lab. The protocol originated in T. Cech's lab. See: Zaug, Kent, and Cech (1984) Science **224**:574-578.]

I. Preparation/Labelling of Primer

1) 5' End-labeling Reaction (see Maniatis p.122, Forward Reaction-Using DNA molecules with protruding 5' termini as template.)

10 pmol primer
1 µl of 10X T4 kinase buffer
10 pmol gamma [^{32}P]ATP (NEN ca. 3000 Ci/mmol, store in -20 ^{0} C walk-in freezer)
10 units T4 polynucleotide kinase (store in -20 ^{0}C walk-in freezer)

---

10 µl

Incubate at 37 ^{0}C for 45 min then shift to 95 ^{0}C for 2 min
Chill on ice 1 min and add 10 µl water.
Extract once with 50% Phenol/48% $CHCl_3$/2% isoamyl alcohol and pipet aqueous phase into scew-capped microfuge tube. (Get some of the phenolic phase to insure recovery of <u>all</u> the aqueous phase.)
Discard phenol into radioactive waste.
Extract the recovered aqueous phase 1X with 1 ml of water-saturated ether. (Chill the tube before pipetting the ether off, the ether is radioactive and chilling will help to prevent any radioactive aerosols.)
Discard the ether into radioactive waste.
Dry the labelled primer in the SpeedVac, using the water aspirator for the vacuum source.
Resuspend the labelled primer in 20 µl of water and store at -20 ^{0}C

in the walk-in freezer.

II. Annealing

1) Annealing Reaction

0.1-0.2 pmol template (8.4 µl of resuspended RNA)
0.3-0.6 pmol labelled primer (1.2 µl of primer)
2.4 µl 5X annealing Buffer

---

12 µl

Heat at 60 °C for 3 min.
Quick chill in a dry ice/ethanol bath for 1 min and place on ice.

III. Reverse Transcriptase Mix

[For 5-6 sets(4 tubes/set) of sequencing reactions]

1 µl undiluted Life Sciences reverse transcriptase (17 units/ul)
5 µl 5X Reverse Transcriptase Buffer
19 µl water

---

25 µl

IV. Sequencing Reactions

Set up four tubes for each annealing mix (A/blue, C/green, G/red or pink and T/yellow). A control tube containing 1X annealing buffer in place of a ddNTP can also be included to indicate nonspecific stops in the sequence.

Each reaction tube should contain:

2 µl annealing mix
1 µl 5X dNTP mix
1 µl 5X ddNTP mix (A, C, G, or T mix or 1X annealing buffer)
1 µl reverse transcriptase mix

5 µl

Incubate at 48 ^{0}C for 30 min

Stop reactions by placing on ice and adding 5 µl of loading dye per reaction tube.

V. Gel

Heat the reaction at 95 ^{0}C for 3 min immediately prior to loading the gel.

Chill on ice after heating at 95 ^{0} C.

Load 5-10 µl per reaction on a pre-warmed 8% acrylamide, 7 M urea sequencing gel.

Load the gel in alphabetical order (A, C, G, T)

Run the gel at 50-55 ^{0}C (This corresponds to about 2000-2500 VDC, ca. 30 mA, on constant voltage.)

A primer of length 16 runs with the bromphenol blue dye, and a fragment of about length 80 should run with the xylene cyanol.

VI. Troubleshooting/Suggestions

1) If background bands are a problem try: (a) an enzyme chase (1 ul reverse transcriptase mix per tube) after 15 min at 48 ^{0}C; or (b) incubation of the reactions at a higher temperature. The enzyme chase usually helps ds DNA sequencing reactions and you can incubate reactions up to 52 ^{0}C.
2) To prevent evaporation of solutions in the tubes, when setting up the various sequencing reactions, keep the tubes on ice and capped.
3) If there is no sequencing ladder either the RNA has been degraded or (more likely) the primer was not end-labelled. In the former case, check the Abs_{260} and $Abs_{260/280}$ of the RNA preps. Two other diagnostic tests for RNA degradation are: a) Run the RNA prep on a 1% agarose gel. The ribosomal RNA bands should be very prominent if there has not been significant degradation. b) Some degradation will always happen. This will show up as nonspecific terminations (actually, run-off products of the primer extension reaction) in the sequencing ladders. If you suspect the latter case, i.e. primer not end labelled, run the end-labelled primer out on a 20% acrylamide/7 M urea gel. The primer should run between the bromphenol blue and xylene cyanol.
4) If the sequencing ladder is there, but weak, either try exposing the gel for a longer period of time (about 10-fold longer, say a week to 10 days) or increasing the amount of RNA in the annealing and sequencing reactions. Use of old phage lysates to make RNA preps may result in poor yields of template. Use fresh (less than 3 months old) lysates to prevent this loss of template recovery.
5) A weak sequencing ladder may also result from inefficient extension of the primer. Dead reverse transcriptase or bad buffers are the two most likely culprits. (Forgetting to add

magnesium to the 5X Reverse Transcriptase buffer would prevent reverse transcriptase from working.)

6) To sequence using DNA templates, try the annealing reaction at 90 ^{0}C for 3 min.
7) For "rare" RNA templates, annealing at 60 ^{0}C for 3 minutes, then at 48 ^{0}C for 30 minutes may give better results.

Sequencing Gel Stock Solutions

38:2 Acrylamide	100-500 ml
10% Ammonium Persulfate	10 ml
TEMED	Use straight from bottle
10X Tris-Borate-EDTA buffer (10X TBE)	500 ml
1X TBE	10-20 liters
2X Loading Dye	10-25 ml

Notes:

1) Acrylamide is a neurotoxin. When weighing acrylamide out, wear gloves and possibly a dust mask. Don't mouth pipet acrylamide solutions.
2) Old TEMED solutions are a common cause of problems that result in non-polymerization of gels. Keep TEMED stored at 4 ^{0}C.
3) Glassware in which filtered solutions are stored or mixed, should be rinsed with water (to rinse out any dust or particulates) before use.

Sequencing Gel Recipes

38:2 Acrylamide

Dissolve
38.0 g acrylamide
2.0 g bis acrylamide
in 80 ml water.
Adjust to 100 ml with water.
Filter through Whatman #1 or #3 filter paper.
Store at 4 °C.

10% Ammonium Persulfate

Dissolve
1 g ammonium persulfate
in 10 ml of water.
Store at 4 °C.
Note: This solution is only good for about 1 week

10X Tris-Borate-EDTA buffer (10X TBE)

	per 100 ml	per 500 ml
0.89 M Tris-Borate	10.8 g Tris-OH	54 g
0.89 M Boric Acid	5.5 g Boric acid	27.5 g
0.02 M EDTA	4.0 ml of 0.5 M EDTA	20 ml
in	80 ml water	400 ml
Adjust to:	100 ml with water	500 ml

Autoclave.
Aliquote into 100 ml bottles and store at 37 °C to prevent precipitation of salts.

1X TBE

	per liter	per 10 liters
0.089 M Tris-Borate	10.8 g Tris-OH	108 g
0.089 M Boric Acid	5.5 g Boric Acid	55 g
0.02 M EDTA	4.0 ml of 0.5 M EDTA pH 8.0	40 ml
	in 1 liter water.	10 liters

Loading Dye

94% Formamide	Add 9.4 ml of deionized formamide (use freshly distilled or deionized, see Maniatis)
0.036 M Tris	
0.8 mM EDTA	0.4 ml of 10X TBE
0.04% Bromphenol Blue	0.1 ml of 4% Bromphenol Blue solution
0.04% Xylene Cyanol	0.1 ml of 4% Xylene Cyanol solution
	10 ml

Store at room temperature.

Sequencing Gels

1) Preparing /Assembling Plates

a) Wash with detergent until clean. It is very important to get any grease spots or smudges off the plates, as these are the primary cause of bubbles forming when pouring gels. Wear gloves when handling clean plates to prevent leaving finger smudges on the plates.
b) Rinse the plates with distilled water, followed by a rinse with 95% EtOH.
c) Let the plates air dry. Cover with a paper towel to keep dust off the surface.
d) Silanize the back (notched) plate with a 5% dimethyldichlorosilane/$CHCl_3$ solution and let dry.
e) Rinse the silanized plate with distilled water and 95% EtOH.
f) Let dry and cover with a paper towel to keep dust off the plate.
g) Clean the spacers and combs with a Kimwipe.
h) Clean any dust off the surface of the plates.
i) Use a small dab of vaseline or grease on the bottom of the

side spacers to help seal the corners of the plates and hold the spacers in place while assembling the plates.

j) Place the top (large square plate) plate dirty side down on a counter, assemble the spacers on the top plate and lay the back (notched) plate, silanized face down, on top of the spacers. (The outside edge of the side spacers must be flush with the edge of the glass plates or the plates will not fit in the buffer chamber of the gel stand.) Try to get the plates as square as possible and use the small clamps to clamp across the bottom and sides of the plates.

k) Cover the top with Saranwrap to keep out dust and set aside until the acrylamide is ready to pour.

2) Preparing the Acrylamide

a) Mix:

15.0 ml water
4.5 ml 10X TBE
9.0 ml 38:2 acrylamide
0.3 ml 10% ammonium persulfate
22.5 g
ultrapure urea

b) Stir or shake, while warming, until all the urea is in solution.

c) Filter through Whatman #1 filter paper into a side arm flask and degas the solution for 10-15 min.

3) Pouring the Gel

a) Pour the degassed acrylamide into a beaker, add 5-10 µl TEMED and gently mix.

b) Pour the acrylamide solution into the assembled plates. If bubbles become trapped between the plates, tilt the plates until the solution uncovers the bubble and then

tilt the plates back while gently rapping the plates with your knuckles. Sometimes the solution flows between the plates more easily if the first few clamps at the top of the plates, along one side, are removed. Removing the clamps will also make inserting the comb between the plates easier.

c) Pour the acrylamide above the top of the notch in the back (notched) plate and insert the comb.

d) Cover the top with Saranwrap and set aside to polymerize (wait several hours before using).

e) If the poured gel is not going to be used immediately, after the gel has polymerized, wrap the top of the gel with a paper towel soaked with 1X TBE, cover with saran wrap, seal by clamping with the large clamps and store in the cold box.

4) Pre-running the Gel

a) Remove all the clamps, the bottom spacer and the comb.

b) Use a syringe to wash out the wells with 1X TBE.

c) Lightly grease the gasket, on the gel stand, with silicone vacuum grease and clamp the plates onto the gel stand.

d) Fill the buffer chambers with 1X TBE and remove bubbles trapped along the bottom of the plates with a stream of 1X TBE squirted through a syringe.

e) Clamp on the metal plates.

f) Attach the power supply leads to the gel stand (-, black, goes to the top and +, red, to the bottom).

g) Adjust the voltage to 1500 VDC and let the gel run until the current has stabilized. Keep increasing the voltage to maintain the current between 20 to 30 ma, the voltage will end up around 2000 to 2500 VDC.

h) Let the gel run until it has warmed up to 50-55 ^{0}C. (Thermometer on front of metal plates.)

5) Loading and Running the Gel

a) Turn off the power supply, unplug the gel stand and remove the metal plate.
b) Use a syringe to wash out the wells with 1X TBE.
c) Use a [^{32}P]-dedicated syringe to load ca. 10 µl into the wells. Wash the syringe several times from two separate beakers of water in between loads.
d) Put the metal plate back on the gel, reattach the power supply leads and turn the power supply on. Run the gel at whatever voltage necessary to keep the current at about 30 ma. (probably about 2000-2500 VDC) which in turn maintains the temperature around 50-55 ^{o}C.

6) Taking the Gel Down

a) Turn off the power supply, remove the leads and remove the metal plate (Do not let the metal plate dip into the bottom buffer reservoir. The buffer in the bottom reservoir is radioactive and once the metal plate is contaminated, it is extremely difficult to decontaminate.).
b) Clean out the upper (non-radioactive) reservoir with a gravy baster and dispose the buffer into the sink.
c) Clean out the bottom (radioactive) reservoir with a gravy baster and dispose the buffer into radioactive waste.
d) Rinse out both reservoirs with water. Flush the wash down the sink.
e) Remove the gel from the gel stand and take the side spacers off the gel.
f) Place the gel, non-silanized (large square plate) plate down, on a counter.
g) Separate the plates (use a razor or thin spatula). Sometimes the gel wants to come off with the (upper) silanized plate, if this happens flip the plates so the

silanized plate is on the bottom.

h) Take a piece of 3mm chromatography paper that has been cut just a little larger than the gel and place the paper square on top of the gel. Carefully roll the paper away from the plate, starting at the bottom of the gel such that the gel comes off onto the paper. (Alternatively, one can use gel-bond, sold by FMC, instead of the paper; use the hydrophilic side of the gel-bond to pick-up the gel.)

i) Wrap the gel in saran wrap (do not trap any bubbles between the gel and the Saranwrap) place on film and expose at -80 ^{0}C. If all went well, the autoradiogram may be developed in 24 hours.

RNA-Sequencing References

Davis, R. W., Botstein, D., Roth, J. R. (1980) *A Manual For Genetic Engineering. Advanced Bacterial Genetics.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory

Hamlyn, P. H., Brownlee, G. G., Cheng, C. C., Gait, M. J., and Milstein, C. 1978. Complete Sequence of Constant and 3' Noncoding Regions of an Immunoglobulin mRNA Using the Dideoxynucleotide Method of RNA Sequencing. *Cell* **15**,1067-1075

Maniatis, T., Fritsch, E. F., Sambrook, J. (1982). *Molecular Cloning* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Rickwood, D., and Hames, B. D. (1982) *Gel Electrophoresis of Nucleic Acids: a practical approach* Eynsham, Oxford; England: IRL Press Ltd.

Zaug, A. J., Kent, J. R., and Cech, T. R. (1984) A Labile Phosphodiester Bond at the Ligation Jnction in a Circular Intervening Sequence RNA. *Science* **224**,574-578.