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Although the synthesis of DNA precursors is closely coordinated with DNA replication, it is still not clear whether deoxyribonucleoside triphosphates (dNTPs) influence DNA replication independently of their interactions with DNA polymerase catalytic sites. In an effort to understand the extent to which rate and fidelity of DNA replication are regulated by its precursors, two distinct mammalian cell mutants, which are defective in deoxyribonucleotide biosynthesis, have been analyzed.

JB3-B is a Chinese hamster ovary (CHO) cell mutant that is temperature-sensitive for DNA replication. Measurement of dNTP pools as a function of time after shift of cultures to the non-permissive temperature revealed that all four dNTP pools declined at similar

rates in extracts of both rapidly isolated nuclei and whole cells. The magnitude and time course of the pool size changes measured in nuclear extracts more closely corresponded to the inhibition of DNA synthesis than did those measured in whole-cell extracts. Ribonucleotide reductase activity was diminished in extracts prepared at the non-permissive temperature, in parallel with the decline in dNTP pool sizes. Moreover, the activity of a cell extract was thermolabile in vitro. The data suggest that the intranuclear concentrations of the four dNTPs determine the rate of DNA replication in JB3-B under the conditions of limited ribonucleotide reductase activity.

Thy- 49 and Thy- 303 are CHO cell mutants that have a mutator phenotype, but they differ in the extents to which spontaneous mutagenesis is stimulated. Both strains have imbalanced dNTP pools that result from loss of allosteric regulation of CTP synthetase. When the DNA precursor pools from S phase-enriched cells were analyzed, large imbalances were seen in the extracts of rapidly isolated nuclei, while whole-cell extracts showed smaller biases, in both strains. The specific nuclear dCTP pool increase produced the large perturbations in the balance of nuclear precursor pools. Thy- 303, which has higher mutation frequencies than Thy- 49, showed the more aberrant precursor pools.

The nuclear pool size changes were correlated with the frequencies of mutations observed in the two strains. The data suggest that the intranuclear balance of DNA precursor pools determines the fidelity of DNA replication.

Insights into Mammalian DNA Replication from Analysis of Mutations Affecting Deoxyribonucleotide Biosynthesis

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Byung-Joo Mun

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Professor of Biochemistry and Biophysics in charge of major

Redacted for Privacy

Head of Department of Biochemistry and Biophysics

Redacted for Privacy

Dean of Graduate School

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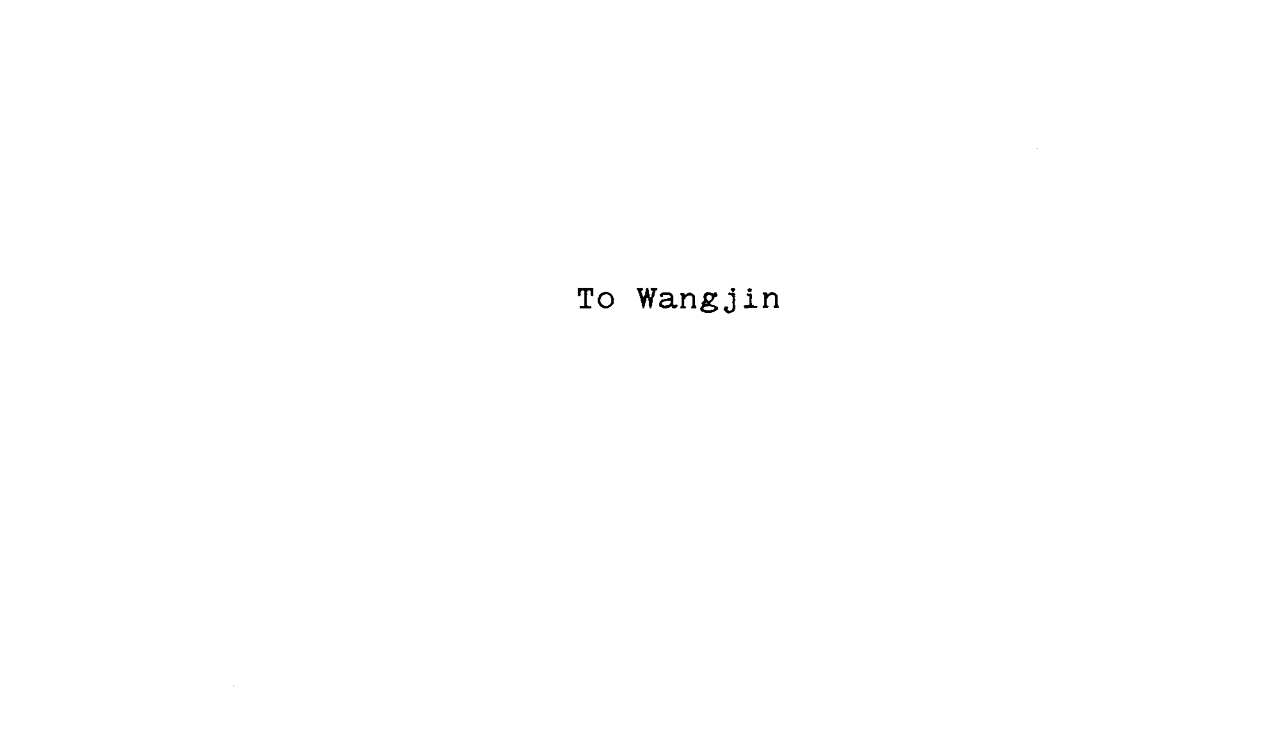

To Wangjin

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Table of Contents

Chapter Page

1 Introduction 1
Regulation of dNTP Biosynthesis 2
De Novo Synthesis 2
Ribonucleotide Reductase 3
CTP Synthetase and Deoxycytidylate Deaminase 5
Salvage, Catabolism, and Substrate Cycles 5
Whole System Control Mechanism during the Cell Cycle 6
Consequences of Perturbations of dNTP Pools 8
dNTPs as Regulators of DNA Replication Fidelity 9
Nature of dNTP Pools 11
dNTP Pool Compartmentation 11
Are DNA Precursors Channeled to Replication Sites? 14
dNTPS as Regulators of DNA Replication Rates 16
Are dNTP Levels Rate-Limiting? 16
Are There Regulatory Proteins that Bind dNTPs? 18

2 A Temperature-Sensitive DNA⁻ Mutant of Chinese Hamster Ovary Cells with a Thermolabile Ribonucleotide Reductase 22
Abstract 23
Introduction 25
Materials and Methods 26
Materials and Reagents 26
Cell Lines and Growth Condition 27
Nuclear Isolation 28
Deoxyribonucleoside Triphosphate Analysis 29
Ribonucleoside Triphosphate Analysis 30
Ribonucleotide Reductase Assay 31
Results 32
Effects of the JB3-B Mutation on Nucleotide Pool Sizes 32
Temperature Sensitivity of Ribonucleotide Reductase in JB3-B Cells 35
Discussion 38
Acknowledgements 44

Table of Contents (continued)

Chapter Page

3 Intranuclear Deoxyribonucleoside Triphosphate Pool Imbalances that are Responsible for the Mutator Phenotype of Thy$^-$ Chinese Hamster Ovary Cells 57
  Abstract 58
  Introduction 60
  Materials and Methods 62
    Materials and Reagents 62
    Cell Lines and Culture Techniques 63
    Isolation of Nuclei 64
    dNTP Pool Measurements 64
    Cell Synchronization 66
    Cell Cycle Analysis 66
  Results 67
    Nuclear and Whole-Cell dNTP Pools in Thy$^-$ Mutator Cell Lines 68
    Cell Cycle Analysis of Synchronized Cells 71
    Intranuclear Pool Imbalances in Thy$^-$ Mutator Cell Lines 73
    Differential Nuclear Compartmentation of the dCTP Pool in Thy$^-$ Mutator Cell Lines 75
    Intranuclear Pool Ratio Perturbations Created by the Increased Nuclear dCTP Pool in Thy$^-$ Mutator Cell Lines 76
  Discussion 79
  Acknowledgement 86

4 Discussion 101
  Intranuclear dNTP Pools as Determinants of DNA Replication Rate 102
  Intranuclear dNTP Pools as Determinants of DNA Replication Fidelity 105
  Conclusion 107

Bibliography 109

Appendices 121
  1: Deoxyribonucleotide Pools as Targets for Mutagenesis by N-Methyl-N-Nitrosourea 121
  2: Ribonucleotide Reductase, A Determinant of 5-Bromodeoxy-Uridine Mutagenesis in Phage T4 123

List of Figures

Figure		Page
1.1	Pathways of deoxyribonucleoside triphosphate biosynthesis in mammalian cells	20
2.1	Mutant (JB3-B) whole-cell and nuclear dNTP pools after temperature shift from 33°C to 39°C	45
2.2	Revertant (3B2R) whole-cell and nuclear dNTP pools after temperature shift from 33°C to 39°C	47
2.3	Mutant (JB3-B) whole-cell rNTP pools after temperature shift from 33°C to 39°C	49
2.4	Ribonucleotide reductase activity in extracts of mutant (JB3-B) and revertant (3B2R1) cells after temperature shift from 33°C to 39°C	51
2.5	Thermolability of ribonucleotide reductase activity from mutant (JB3-B) and revertant (3B2R) cells	53
2.6	Heat sensitivity of ribonucleotide reductase activity from transfectant (206) cells	55
3.1	Metabolic alterations in Thy^- mutants	87
3.2	Nuclear dNTP pools versus time of treatment with NP-40-containing nuclear isolation buffer	89
3.3	Nuclear and whole-cell dNTP pools from non-synchronized wild-type and Thy^- mutant cells grown in 5 μM thymidine	91
3.4	DNA distribution of synchronized wild-type and Thy^- mutant cells	93
3.5	Nuclear and whole-cell dNTP pools from synchronized, S-phase wild-type and Thy^- mutant cells grown in 5 μM thymidine	95

List of Figures (continued)

Figure Page

3.6 Nuclear and whole-cell dNTP pools from S-phase wild-type and Thy^- mutant cells grown in 100 μM thymidine 99

List of Tables

Page

3.1 Proportion of nuclear pool to the whole-cell dNTP pool in S-phase cells and non-synchronized cultures 97

3.2 Perturbations of nuclear and whole-cell pool ratios in S-phase cells grown in 5 μM thymidine 98

Insights into Mammalian DNA Replication From Analysis of Mutations Affecting Deoxyribonucleotide Biosynthesis

CHAPTER 1

Introduction

Although direct roles of deoxyribonucleoside triphosphates (dNTPs) in regulating DNA replication have not been established, the synthesis of DNA precursors is closely coordinated with DNA replication in mammalian cells. The cell needs a continuous and balanced supply of the four dNTPs to replicate its DNA properly. Clearly this requires a tight regulation for the synthesis of dNTPs. The regulation is achieved by a combination of several different mechanisms, including allosteric properties of the regulated enzymes, interconversion of active and inactive forms of ribonucleotide reductase, substrate cycles, and control of enzyme synthesis during the cell cycle.

The biological importance of these regulatory mechanisms become apparent from the numerous severe genetic consequences of disturbing the normal balance, ranging from increased point mutations to cell death by

thymidine deprivation. In particular, the increase in spontaneous mutagenesis suggests the effect of dNTP pool imbalances on the fidelity of DNA replication.

Control of replication rates by DNA precursors is probably somewhat indirect. A multiprotein complex, consisting of enzymes involved both in dNTP synthesis and DNA replication in the nucleus, has been postulated to help to efficiently deliver precursors to replication sites. However, in mammalian cells, dNTPs have been found to be synthesized in the cytoplasm, with subsequent passage into the nucleus to participate in DNA synthesis. Moreover, it seems likely that the eukaryotic replication apparatus is normally saturated with dNTPs, because DNA polymerases α and δ have very low K_M values for dNTPs. However, some control, like substrate-level control of DNA polymerase activity, may be exerted through low pool sizes of dGTP. Other regulation through possible interaction of dNTPs with sites other than catalytic sites on DNA polymerase has been also proposed.

Regulation of dNTP Biosynthesis

De Novo Synthesis

Figure 1.1 gives an overview of dNTP synthesis. The main route for dNTP synthesis is the de novo path-

way, in which ribonucleotides are formed first from small molecules and subsequently reduced to deoxyribonucleotides. Three enzymes are subject to allosteric control and therefore regulatory targets for the maintenance of appropriate dNTP pool sizes. Ribonucleotide reductase, an enzyme that occupies a key position in dNTP synthesis, reduces all four ribonucleoside diphosphates to the corresponding deoxyribonucleotides and provides the cell with a balanced supply of the four dNTPs. Two other regulated enzymes, deoxycytidylate deaminase and CTP synthetase, help to maintain the balance between the two pyrimidine dNTPs.

Ribonucleotide Reductase

Great interest has focused upon ribonucleotide reductase because it catalyzes the first committed step in DNA replication, providing the main supply of dNTPs required for DNA synthesis. The mammalian enzyme consists of two nonidentical subunits, M1 and M2 (Hopper, 1972; Thelander *et al.*, 1980). The M1 protein is a dimer of molecular weight around 170,000 (Thelander *et al.*, 1980) and protein M2 is also a dimer with a molecular weight of close to 90,000 (Thelander *et al.*, 1985). While the large subunit contains the allosteric effector binding sites, a tyrosyl free radical essential to catalytic activity is present in the small subunit.

This tyrosyl free radical may be a target for the regulation of the enzyme activity. M2 subunit can be isolated in an active and inactive form: Active M2 contains an organic free radical, localized to a specific tyrosyl residue of the polypeptide chain, while inactive M2 contains a normal tyrosine (Gräslund *et al.*, 1982; Larsson and Sjöberg, 1986). A possible mechanism, which controls the generation of tyrosyl radicals within existing, radical-free, inactive M2 molecules and thereby its catalytic activity, has been proposed (Thelander *et al.*, 1983), although the nature of the control has not yet been elucidated.

The complex allosteric regulation is responsible for the balanced supply of all four dNTPs (Thelander and Reichard, 1979; Reichard, 1987). Two distinct regulatory sites have been defined: the activity site, which binds ATP and dATP and regulates the overall catalytic activity of the enzyme; and the specificity site, which binds ATP, dATP, dGTP, and dTTP and controls its substrate specificity (Eriksson *et al.*, 1981; Thelander and Reichard, 1979). However, ribonucleotide reductase is not subject to regulation distinguishing between the specificity for the two pyrimidine ribonucleotides. Instead, the proper intracellular balance between dCTP and dTTP is maintained by two other allosteric enzymes.

CTP Synthetase and Deoxycytidylate Deaminase

Sites for allosteric control for the pyrimidine nucleotide synthesis are CTP synthetase and dCMP deaminase. While CTP synthetase regulates the relative amounts of the pyrimidine ribonucleotides, dCMP deaminase controls the balance between the pyrimidine deoxyribonucleotides. The latter enzyme was also found to provide most of the dUMP required for dTTP formation when thymidine and deoxyuridine are absent from the medium (Jackson, 1978; DeSaint Vincent *et al.*, 1980).

Salvage, Catabolism, and Substrate Cycles

In addition to the main de novo route, two side routes exist. While the main route is a one-way street, each side route leads in both directions, with material entering and leaving dNTP pools. Kinases catalyze the phosphorylation of deoxynucleosides via salvage pathways. These kinases, together with nucleotidases, enzymes that dephosphorylate nucleotides, form substrate (futile) cycles, thus providing an additional control system for the balance between degradation and resynthesis and of the pyrimidine dNTPs. Evidence for this regulation was accumulated in recent work from Reichard's laboratory (Bianchi *et al.*, 1986b; Nicander and Reichard, 1985a; 1985b). The ratio between these two activities was shifted toward anabolism when dNTPs

were short in supply, e.g., when de novo synthesis was blocked in cells treated with hydroxyurea (Bianchi *et al.*, 1986b). Catabolism was favored when dNTPs accumulate, e.g., when DNA synthesis is inhibited by aphidicolin, an inhibitor of DNA polymerase α (Nicander and Reichard, 1985a, 1985b). The activities of the kinases are allosterically regulated by their respective dNTPs (Cheng, 1978; Bohman and Eriksson, 1988), while the activities of nucleotidases were found to largely depend on nucleotide concentration (Bianchi *et al.*, 1986b; Nicander and Reichard, 1985a).

In addition to the above-listed mechanisms, the activity of the dNTP metabolic system as a whole is controlled during the cell cycle.

Whole System Control Mechanism during the Cell Cycle

The sizes of dNTP pools vary during the cell cycle, with the largest pool sizes found during S phase and the smallest in G0 (Walters *et al.*, 1973; Skoog *et al.*, 1973; Reichard, 1985). Parallel changes in the activities of the enzymes of dNTP synthesis were noted in early studies (Elford *et al.*, 1970; Nordenskjold *et al.*, 1970; Rode *et al.*, 1980), as well as in much subsequent work. Many of the enzymes, both those catalyzing the de novo pathway and those participating in substrate cycles, showed cell cycle-dependent variations

of their activities. Recently, the availability of cDNA probes for these enzymes (Caras *et al.*, 1985: Thelander and Berg, 1986) has led to study of the molecular mechanisms responsible for the varied activities of the enzymes. Evidence for genetic control was found, which regulates the amount of enzyme proteins or mRNAs and thereby the concentrations of the enzymes, during the cell cycle.

The variation in ribonucleotide reductase activity depends on the synthesis and degradation of the M2 protein. The level of M2 subunit varies with a half-life of 3 hours in mouse mammary tumor TA 3 cell line (Eriksson *et al.*, 1984a), while the amount of M1 subunit remains constant and in excess throught the cell cycle, with a half-life of at least 15 hours in bovine kidney MDBK cells (Engström *et al.*, 1985; Mann *et al.*, 1987). Thus, the two subunits are differentially regulated during the cell cycle (Eriksson and Martin, 1981; Engström *et al.*, 1985; Engström and Rozell, 1988), and M2 levels correlate closely with both enzyme activity and DNA synthesis. Changes in thymidine kinase activity are also controlled by these two post-transcriptional mechanisms (Groudine and Casmir, 1984; Gross and Merrill, 1988; James and Thomas, 1988). However, thymidylate synthase is an example of an enzyme whose mRNA level increases when cells are stimulated to

enter S phase (Jenh *et al.*, 1985; Ayusawa *et al.*, 1986).

Consequences of Perturbations of dNTP Pools

Faulty operation of some of these regulatory mechanisms has severe genetic consequences. The numerous effects of dNTP pool imbalances have been described, including increased mutagenesis, recombinogenesis, chromosomal abnormalities, induction of latent viruses, and cell death, suggesting the importance of the precise regulation of the intracellular dNTP concentrations (DeSerres, 1985; Kunz, 1988). Two extreme cases, leading to cell death, were the effects of severe dTTP deprivation (thymineless death) and certain immune disease accompanied by accumulation of dATP or dGTP. The latter situation may lead to a depletion of other dNTPs via allosteric effects on ribonucleotide reductase. Under less extreme pool bias conditions, many kinds of genetic abnormalities were found, ranging from increased mutagenesis to sister chromatid exchange. However, from a mechanistic standpoint, most attention has focused upon mutagenesis induced by dNTP pool imbalances. Thus, it is appropriate to consider roles of deoxyribonucleotides as regulators of the accuracy of DNA replication.

dNTPs as Regulators of DNA Replication Fidelity

In vitro, the fidelity of DNA replication by various DNA polymerases is strongly affected by the imbalanced dNTP pools in the incubation mixture (Fersht, 1979; Kunkel and Loeb, 1981; Abbotts and Loeb, 1985; Petruska and Goodman, 1985; Kunkel *et al.*, 1986). Not only is there an increased misincorporation of a nucleotide present in excess, but a next nucleotide effect also arises, in which a high concentration of the next nucleotide promotes its incorporation, thereby blocking the 3'-exonucleolytic proofreading of previously misincorporated nucleotide (Fersht, 1979).

In vivo, dNTP pool biases brought about by addition of deoxyribonucleosides to the medium were reported to increase mutation rates of cultured cells (Bradley and Sharkey, 1978; Meuth, 1981). Mutations affecting the enzymes involving in dNTP metabolism, notably CTP synthetase (Trudel *et al.*, 1984), ribonucleotide reductase (Weinberg *et al.*, 1981) and dCMP deaminase (Weinberg *et al.*, 1985) were found to act as mutator genes. The resulting derangements of dNTP pools were accompanied by up to a few hundredfold increase in spontaneous mutation rates at some genetic loci. In addition, DNA sequence analysis of the mutations induced in a CHO cell mutant, that is defective

in feedback control of CTP synthetase, has suggested that the mutator phenotype is due to replication errors induced by the dNTP pool bias, incorporation errors and next nucleotide effects (Phear *et al.*, 1987; Phear and Meuth, 1989).

However, the relationship of the dNTP pool imbalances to DNA synthesis in mammalian cells is still somewhat obscure since enhanced mutagenic effects are not always accompanied by dNTP pool size changes. For example, in mouse lymphosarcoma cells the mutation of the ribonucleotide reductase M1 subunit conferring resistance to the inhibitor dATP enhanced spontaneous mutaion rate with elevations in the dATP and dGTP pools (Weinberg *et al.*, 1981). However, when the gene for the modified M1 subunit was cloned and introduced into wild-type CHO cells, the introduction did not disturb dNTP pool sizes, but it did result in increased mutation rate (Caras and Martin, 1988). Moreover, a dCMP deaminase deficiency, which causes dCTP accumulation and dTTP depletion, is not mutagenic in all cell lines (A.L. Arecco and C.K. Mathews, unpublished data). Thus, it seems likely that the increase in mutation frequency can be uncoupled from the altered pool balance. However, since all the available data are based on dNTP pool sizes in non-synchronized whole cells,

conclusions drawn from pool measurements would be complicated by the dNTP compartmentation effects.

Nature of dNTP Pools

dNTP Pool Compartmentation

How are dNTPs distributed within the compartments of eukaryotic cells? Do the dNTPs form physically distinct pools within a cell? One clear-cut case of compartmentation concerns dNTPs of mitochondria, substrates for the mitochondrial DNA polymerase. Our laboratory (Bestwick *et al.*, 1982; Bestwick and Mathews, 1982) showed that because of permeability barries, mitochondria contain physically and kinetically distinct dNTP pools. However, the small size of these pools makes this problem relatively minor. Of more importance is the distribution of dNTPs between cytoplasm and nucleus, the cell compartment where DNA replication is taking place. To date, only few laboratories have attempted to measure separate nuclear and cytoplasmic pools. However, because of the porous nature of the nuclear membrane, conventional subcellular fraction procedures almost certainly redistribute small molecules between nuclear and cytoplasmic compartments. This problem was partly circumvented by Skoog and Bjursell (1974), who isolated CHO cell nuclei

by fractionation of nuclei in nonaqueous solvents. Our laboratory developed a rapid method (approximately 20 sec) for isolating nuclei free of cytoplasmic contamination (Leeds *et al.*, 1985). The method involves a ten-second NP-40 detergent treatment followed by a five-second wash. The dNTP values for these rapidly isolated nuclei were stable over washing intervals of 30 seconds in that study and over detergent treatment times of up to 2 minutes in my study (chapter 3). It seems likely, therefore, that the dNTP levels we measured in the nuclear extracts represent true intranuclear pool values. When Leeds and Mathews (1987) compared in CHO cells the kinetics of dNTP pools of labeling by exogenous nucleosides in nuclei and whole cells, no difference were seen, suggesting that the nuclear and cytoplasmic pools constitute a single metabolic compartment. However, the process by which dNTPs pass through the nuclear membrane is yet to be defined.

In addition to the physical compartmentation mentioned above, compartmentation of dNTP pools may also occur between cells (intercellular) or within cells (intracellular). Using synchronized cell populations, Leeds and Mathews (1987) demonstrated that Chinese hamster ovary (CHO) cells incorporated deoxycytidine much more efficiently into dCTP during G1 than during S phase. Since G1 phase cells are not replicating their

DNA (except for mitochondrial DNA), the pool that is derived from deoxycytidine was interpreted as a replication-excluded dCTP pool, focusing attention on intercellular compartmentation. These results were extended with mouse S49 cells to deoxyadenosine (Duan and Sadée, 1987). This is a form of intercellular, not intracellular, compartmentation, with the replication-inactive pools being formed in non-S-phased cells.

Evidence for intracellular compartmentation of dNTPs within cells was also obtained from isotope experiments. While the dTTP pool in mouse 3T6 cells is a single kinetic pool (Nicander and Reichard, 1983), there is strong evidence for compartmentation of both dCTP and dGTP pools. For dCTP, isotope flow kinetics in 3T6 cells suggested that one pool, labeled from cytidine, was preferentially used for DNA synthesis, while the second pool, labeled from deoxycytidine, was preferentially used for the synthesis of deoxyliponucleotides--the deoxycytidine analogs of CDP-choline and CDP-ethanolamine (Nicander and Reichard, 1983; Spyrou and Reichard, 1987, 1989). Evidence for compartmentation for the dGTP pool was presented with mouse S49 lymphoblasts (Nguyen and Sadée, 1986). One pool used preferentially for DNA replication arose by reduction of GDP and differed from a second pool arising from the phosphorylation of deoxyguanosine. A similar situation

may also occur with dATP (Duan and Sadée, 1987). Whether compartmentation is intra- or inter-cellular, there appears to be general agreement that dNTPs (except dTTP) formed by the action of ribonucleotide reductase via the de novo pathway are more readily utilized for incorporation into DNA than those formed by salvage pathways.

Are DNA Precursors Channeled to Replication Sites?

Multienzyme complexes were suggested as a possible explanation for dNTP compartmentation. According to this concept, DNA replication is coordinated with synthesis of its precursors through the existence of physically associated mutienzyme complexes. dNTPs synthesized by the complex are channeled to the replication fork and preferentially incorporated into DNA, protected from mixing with outside dNTPs. Thus, within the complex the dNTPs are assumed to form a separate compartment, maintained at concentrations higher than those dNTPs far from replication sites.

Such a model was originally supported from the data accumulated on the T4-phage-infected *E. coli* system in our laboratory and in Greenberg's laboratory (reviewed by Mathews, 1985, 1988; Mathews *et al.*, 1988, 1989). Since 1980, several laboratories have described multienzyme aggregates from mammalian cells (Reddy and

Pardee, 1980, 1982, 1983; Wickremasinghe *et al.*, 1983; Ayusawa *et al.*, 1983a; Harvey and Pearson, 1988), and the putative dNTP-DNA synthesizing complex, named replitase, has also been postulated to be assembled in the nucleus when cells enter S phase (Reddy and Pardee, 1980, 1982; Hammond *et al.*, 1989).

However, several observations argue against channeling of DNA precursors to replication sites in mammalian cells (reviewed by Mathews and Slabaugh, 1986). First, intracellular dNTP concentrations seem to exceed those needed to saturate replicative polymerases (Dresler *et al.*, 1988; Leeds *et al.*, 1985). In addition, in vitro DNA replication systems from eukaryotic cells have higher affinities for dNTP substrates than do prokaryotic systems (Mathews, 1985). However, eukaryotic replication rates are slower than those in prokaryotes by at least an order of magnitude (Kornberg, 1980, 1982). Second and more direct are recent findings on the cytoplasmic localizations of dNTP biosynthetic enzymes, including ribonucleotide reductase, thymidylate synthetase, and dUTpase (Leeds *et al.*, 1985; Engström *et al.*, 1984; Kucera and Paulus, 1986; Vilpo and Autio-Harmainen, 1983; Wawra, 1988), while DNA polymerase α resides in the nucleus (Bensch *et al.*, 1982). At the same time, studies on the rate of labeling of dNTP pools by exogenous nucleosides (Leeds and

Mathews, 1987) suggest that dNTPs are synthesized in the cytoplasm and then transported into the nucleus without establishment of a concentration gradient. Recent work of Wawra (1988), involving microinjection of exogenous radiolabeled DNA precursors, lead to a similar conclusion.

Therefore, this model can hardly be accepted in its original form. However, a multienzyme complex comprising enzymes involved in dNTP synthesis (but not also DNA replication) and some form of channeling of small molecules during the synthesis of dNTPs remain an attractive possibility. Mathews and Slabaugh (1986) have suggested that the rough endoplasmic reticulum associated with nuclear membrane might then serve as a vehicle for transport of newly synthesized cytoplasmic dNTPs into the nucleus. However, the dNTP channeling model is difficult to test rigorously, because it invokes weak interactions among enzymes--interactions that may occur only at the very high protein concentrations found inside cells (Fulton, 1982).

dNTPS as Regulators of DNA Replication Rates

Are dNTP Levels Rate-Limiting?

The most direct way for DNA replication rates to be controlled by DNA precursors would be simple

substrate-level control of DNA polymerase activity, by cell cycle-related dNTP pool sizes which result from cell cycle-dependent variations of dNTP biosynthetic enzyme activities. The fact that the sizes of all four dNTP pools increase as cycling cells approach S-phase strongly suggests the existence of substrate-level control.

In general, dNTP pool determinations from many types of cells suggested that average intracellular dNTP concentrations are relatively low--in 10 to 100 μM range, when pool sizes are divided by average intracellular volumes. However, saturating concentrations for replicative DNA polymerases were observed not to be consistent because interpretation of the data is complicated by several factors. For example, earlier work on the substrate concentration dependence of permeablized cell systems suggested that dNTP levels in the range of 50 to 100 μM were needed to saturate the replication machinery (reviewed by Mathews, 1985). Since estimated dNTP concentrations fall below these values, it suggests that replication rates are determined by dNTP concentrations. However, recent studies (Dresler et al., 1988) on concentration dependence, both of replicative polymerase (α and δ) and of permeabilized cells, have yielded lower values than most of those previously reported, suggesting that mammalian

replication sites are supplied with dNTPs at saturating concentrations for replicative DNA polymerases.

However, more attention has focused upon dGTP levels. In nearly every cell analyzed, dGTP is the least abundant of the four dNTPs, with pool sizes one to two orders of magnitude lower than those of the most abundant dNTPs. Estimates of intranuclear dGTP levels in S-phase CHO cells were found in our laboratory to be in the order of 10 μM, which may fall below the level needed to saturate the replicative apparatus; a similar possibility was suggested by data of Dresler et al. (1988). In permeable human fibroblasts, three of the four dNTPs had concentrations well above the K_M, while the fourth, dGTP had a cellular concentration quite close to the K_M. These findings invoke the possibility that channeling of deoxyribonucleotides (at least for dGTP) may be necessary for the attainment of high rates of DNA replication. However, the channeling model currently has little support in eukaryoyic cells, as discussed above. Thus, it is possible that dGTP plays a role in regulating DNA replication through substrate limitation.

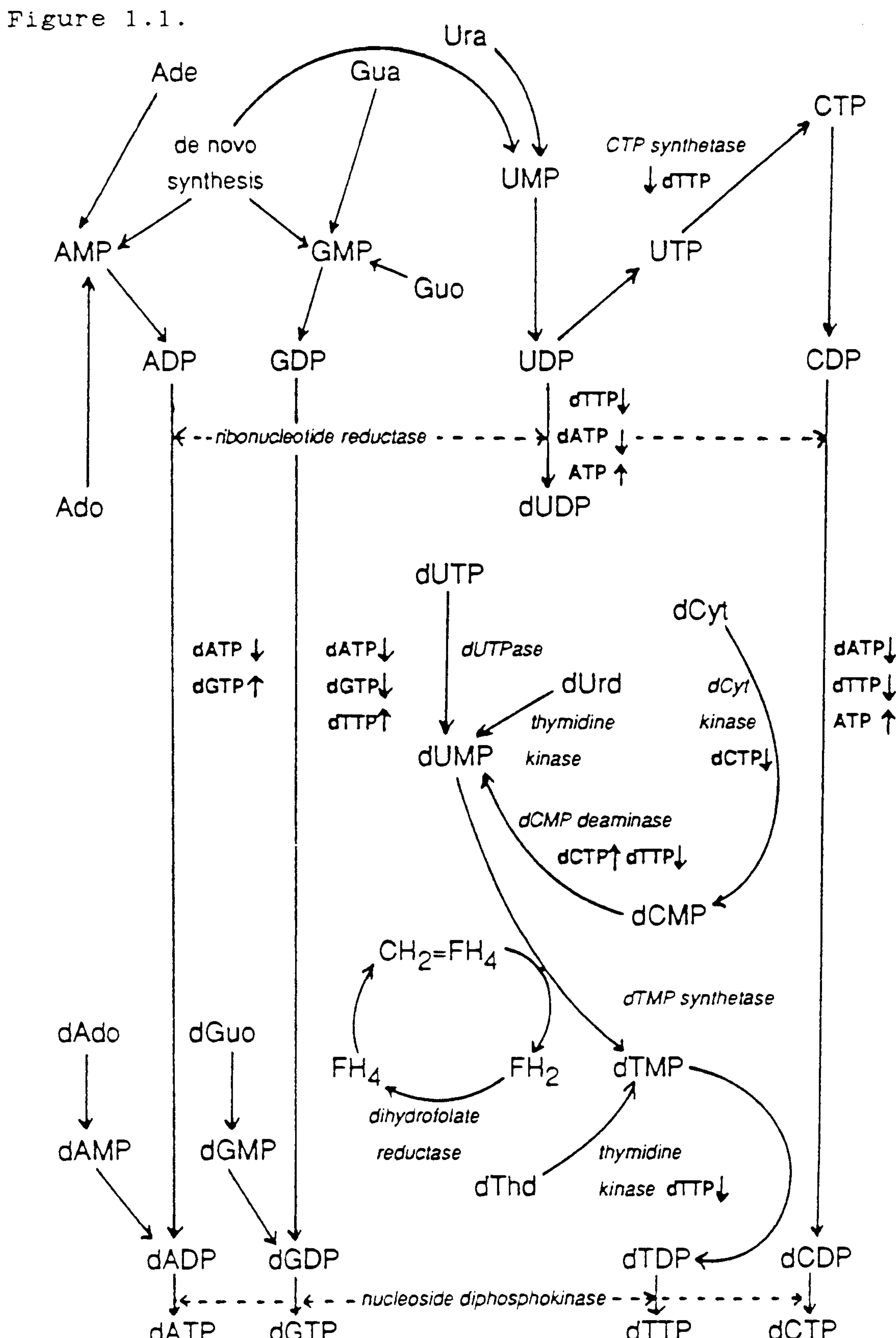
Are There Regulatory Proteins that Bind dNTPs?

While the data mentioned above support substrate-level control of DNA replication in eukaryotic cells,

there remains the possibility of a subtle regulatory mechanism through interaction with sites other than catalytic sites on DNA polymerases. Evidence published in early years suggested that DNA polymerase α contains regulatory binding sites for dNTPs independent of the catalytic site (Steinberg et al., 1979; Grindey *et al.*, 1980). At the same time, inhibition of DNA polymerase α by aphidicolin was reported to be competitive specifically with dCTP (Huberman, 1981). To rationalize this apparent anomaly, Nicander and Reichard (1981) proposed that dCTP regulates DNA polymerase α activity by binding to an allosteric site, and that aphidicolin inhibits by binding to this site, not the catalytic site. Later, Wierowski et al.(1983) described a nucleoside triphosphate-binding site through which binding of nucleotides stimulated polymerase α activity. More recently, Diffley (1988) used affinity labeling to demonstrate two dNTP-binding sites in polymerase α-primase complex, and he suggested that one of these is a regulatory site. Thus, dNTPs seem to serve as potential regulators as well as substrates of DNA polymerases.

Figure 1.1. Pathways of deoxyribonucleoside triphosphate biosynthesis in mammalian cells.

Figure 1.1.



CHAPTER 2

A Temperature-Sensitive DNA$^-$ Mutant of Chinese Hamster Ovary Cells with a Thermolabile Ribonucleotide Reductase

Running title: *ts* Ribonucleotide Reductase CHO Cell Mutant

Byung-Joo Mun and Christopher K. Mathews
Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

Author phone number: (503) 737-4511

Abstract

JB3-B is a Chinese hamster ovary cell mutant previously shown to be temperature-sensitive for DNA replication (J.J. Dermody, B.E. Wojcik, H. Du, and H.L. Ozer, Mol. Cell. Biol. *6*, 4594-4601 (1986)). Because preliminary evidence suggested that the defective function involved DNA precursor biosynthesis, we investigated the biochemical basis responsible for this mutation. Measurement of deoxyribonucleoside triphosphate (dNTP) pools as a function of time after shift of cultures from 33°C to 39°C revealed that all four dNTP pools declined at similar rates in extracts prepared either from whole cells or from rapidly isolated nuclei. Ribonucleoside triphosphate pools were unaffected by a temperature shiftup, ruling out the possibility that the mutation affects nucleoside diphosphokinase. However, ribonucleotide reductase activity, as measured in extracts, declined after cell cultures underwent a temperature shiftup, in parallel with the decline in dNTP pool sizes. Moreover, the ribonucleotide reductase activity in a cell extract was thermolabile *in vitro*, consistent with the model that the JB3-B muation affects the structural gene for one of the ribonucleotide reductase subunits. However, the

possibility of an indirect effect on ribonucleotide reductase activity in JB3-B has not been excluded since ribonucleotide reductase was temperature-stable in the cells transfected with human sequences other than those encoding the subunits of this enzyme.

Introduction

In prokaryotic organisms conditional-lethal mutations affecting DNA replication have been enormously useful in identifying proteins essential to the synthesis of DNA and its precursors (Kornberg, 1980; 1982). Mutations that specifically affect the synthesis of deoxyribonucleoside triphosphates (dNTPs) have helped to clarify metabolic and genetic relationships between deoxyribonucleotide synthesis and DNA replication (Reichard, 1988; Sargent and Mathews, 1987). For example, mutations in the genomes of both *Escherichia coli* and bacteriophage T4 have been used to generate evidence supporting a direct physical connection between the replication apparatus and the enzymatic machinery for dNTP synthesis (Mathews *et al.*, 1988).

By contrast, few DNA-defective mutants are available in mammalian cells, a fact that limits the use of genetic approaches to an understanding of their DNA metabolism (Dermody *et al.*, 1986; Ozer *et al.*, 1987). Nine such mutants were described by Dermody *et al.* (1986), as a result of biochemical screening of temperature-sensitive (*ts*) growth mutants of Chinese hamster ovary (CHO) cells. They exhibited rapid inhibition of DNA synthesis but not protein synthesis upon

shift of growing cultures from the permissive temperature (33°C) to the non-permissive temperature (39°C). Experiments that tested the ability of cells to support the DNA replication of polyoma and adenovirus at 39°C suggested that the *ts* muation affected the synthesis of DNA precursors in just one of these nine mutants, JB3-B (Dermody *et al.*, 1986). No such *ts* mutants had previously been described for mammalian cells. We consequently investigated the nature of the biochemical defect in this mutation. The mutant cells were found to contain a thermolabile form of ribonucleotide reductase, a finding that confirms the putative defect in DNA precursor metabolism. However, the temperature-stability of the ribonucleotide reductase observed in transfectant cells, which did not acquire the DNA sequences for this enzyme's subunits, raises the possibility that the primary defect may not be in a gene encoding the ribonucleotide reductase subunits.

Materials and Methods

Materials and Reagents

Radioactively labeled deoxyribonucleoside 5′-triphosphates obtained from ICN Pharmaceuticals were: [8-^{3}H]dATP (22 Ci/mmol), [methyl-^{3}H]dTTP (22 Ci/mmol), [5-^{3}H]dCTP (25 Ci/mmol), and [8-^{3}H]dGTP (11 Ci/mmol).

[5-^{3}H]cytidine 5'-diphosphate (25.3 Ci/mmol) was purchased from New England Nuclear. Unlabeled nucleotides and adenylylimido-diphosphate (β,γ-imidoadenosine 5'-triphosphate; AMP-PNP) were obtained from Sigma Chemical Company. The alternating copolymers poly (dA-dT) and poly (dI-dC) were from Pharmacia. Purified *E. coli* DNA polymerase I (6600 U/mg protein) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Boehringer-Mannheim. Tri-N-octylamine was obtained from ICN Pharmaceuticals. Freon-113 (1,1,2-trichlorotrifluoroethane) was from Aldrich Chemical Company. Nonidet P-40 (NP-40) was obtained from Particle Delta Laboratories.

Cell Lines and Growth Condition

The mutant JB3-B, and its revertant 3B2R and transfectant 206 (Dermody *et al.*, 1986; Ozer *et al.*, 1987) used in these experiments were obtained from Dr. Harvey L. Ozer (New Jersey College of Medicine and Dentistry). JB3-B cells are *ts* DNA$^-$ mutants isolated from CHO-S cells, which show a rapid inhibition of DNA synthesis without concomitant inhibition of protein synthesis upon shift from 33°C to 39°C (Dermody *et al.*, 1986; Ozer *et al.*, 1987). 3B2R cells are non-temperature-sensitive revertant cells isolated from *ts* JB3-B on the basis of spontaneous colony formation at

39°C (Dermody *et al.*, 1986; Ozer *et al.*, 1987). 206 cells are a transfectant line of JB3-B, in which the *ts* defect has been corrected with sequences from a human cosmid library (B.E. Wojcik, Ph. D. Thesis, City University of New York, 1988). All three cell lines were maintained in monolayer culture at the permissive temperature of 33°C in Dulbecco's modified Eagle medium (Irvine Scientific) with proline, supplemented with 10% fetal bovine serum (GIBCO)and penicillin-streptomycin (GIBCO). The restrictive temperature for these studies was 39°C. The *ts* mutant cells were used within a few passages from frozen storage and never allowed to reach confluency before use.

Nuclear Isolation

We used the rapid technique developed in this laboratory (Leeds *et al.*, 1985) for isolation of nuclei from monolayer cultures. The method requires less than twenty seconds for isolation of washed nuclei. Briefly, each 100-mm plate was placed on ice, and the medium was removed by aspiration. The cells were lysed by addition of 2.5 ml of 1% NP-40 detergent in nuclear isolation buffer (Leeds *et al.*, 1985). After ten seconds of rocking the plate in the presence of the buffer, the buffer was aspirated and the cells washed for five seconds with 5.0 ml of ice-cold phosphate-

buffered saline (PBS). Following aspiration of the PBS, the nuclei remaining on the plate were immediately extracted for analysis of dNTP content. Whole-cell monolayers growing in the same medium were treated identically, except for omission of the nuclear isolation treatment.

Deoxyribonucleoside Triphosphate Analysis

Both whole cells and nuclei were extracted for nucleotide analysis by the two-step extraction procedure of North *et al.* (1980) with the following modifications: 5% trichloroacetic acid was used in place of 5% perchloric acid for the second step of the extraction, and acid extracts were neutralized with tri-N-octylamine as described by Garrett and Santi (1979). The contents of three 100-mm culture dishes were pooled for whole-cell assays, and the contents of four dishes were pooled for nuclear assays. dNTP pool sizes were determined by the DNA polymerase-based enzymatic assay (North *et al.*, 1980), which measures incorporation of a limiting dNTP into an alternating copolymer template [poly (dA-dT) or poly (dI-dC)] by DNA polymerase I in the presence of an excess of the labeled complementary dNTP. An additional change was made in the assay procedure: 100 mM dAMP was added to each reaction mixture to limit product breakdown. Aliquots from each assay

reaction mixture were taken for measurement at three different times of incubation, to insure that plateau incorporation values were always reached. Also, for each assay two different amounts of extract were assayed, to insure that cpm incorporated was directly proportional to volume of extract assayed. The values were corrected for both the background incorporation and the sample incorporation for dilution of the specific activity of added radioactive dNTPs by the sample. Results are expressed as pmol dNTP/10^6 whole-cells or 10^6 nuclei.

Ribonucleoside Triphosphate Analysis

The whole-cell extracts used for dNTP assays were also analyzed for ribonucleoside triphosphate (rNTP) content by high performance liquid chromatography utilizing a Varian high pressure liquid chromatograph (Model 5000) coupled with a Vista data analysis system (Model 401). Aliquots (50 μl) of each extract were analyzed on a strong anion exchange column (Partisil 10-SAX, 4.6 mm x 25 cm, Whatman) plus a guard column of the same material. The chromatography was performed in an isocratic mode at 30°C with a flow rate of 1.5 ml/minute. The eluant was 0.35 M potassium phosphate buffer, pH 3.85. The UV detector operated at 254 nm. The rNTPs in the cell extracts were identified by com-

paring retention times of the unknown peaks with those of standard rNTPs chromatographed under the same conditions just before the sample under observation. The amount of each rNTP was determined by calculating the peak area and reading the concentration from the standard curve. Results are expressed as nmol rNTP/10^6 cells.

Ribonucleotide Reductase Assay

Preparation of the enzyme extracts and assays of ribonucleotide reductase were performed as previously described by this laboratory (Slabaugh *et al.*, 1984) with minor modifications. Exponentially growing cells were seeded at a density of 2×10^6 cells per 100-mm culture dish, incubated at 33°C for 40 hours, and shifted to 39°C. At the indicated time points, the cells were collected and the enzyme extracts were prepared as described by Slabaugh *et al.* (1984), except that 2 mM $MgCl_2$, 20 μM $FeCl_3$ and 20 μM PMSF were added to the lysis buffer. Aliquots of 5×10^6 cells were assayed for intracellular CDP reductase activities. Aliquots from each assay reaction mixture were taken for measurement at three different times of incubation, to ensure that the activity of the enzyme was linear with respect to the incubation time. All assays were carried out in duplicate. The duplicate assays agreed

within 10% of each other. Enzyme activity is expressed as picomoles per milligram of protein.

To determine the thermolability of the enzyme, 20 μl of enzyme preparation was mixed with dithiothreitol, 40 mM $FeCl_3$, 4 mM magnesium acetate and 40 μM CDP and then brought to the desired temperature by placing the tubes in a water bath at 39°C and 42°C. At the indicated times, the samples were removed from the water bath and immediately cooled on ice. The remaining enzyme activity was then measured by incubation for 30 minutes (0, 15, 30 min) at 33°C after addition of 110 μM CDP and [^{3}H]CDP (100 cpm/pmol), and HEPES and AMP-PNP at the final concentrations of reaction mixture for CDP reduction (Slabaugh *et al.*, 1984).

Results

Effects of the JB3-B Mutation on Nucleotide Pool Sizes

Experiments involving inhibition of viral DNA synthesis and cell cycle kinetics suggested the possibility of a defect in deoxynucleotide metabolism in JB3-B (B.E. Wojcik, Ph. D. Thesis, City University of New York, 1988). Preliminary experiments indicated that, following shift of a JB3-B culture from 33°C to 39°C, one or more dNTP pools declined, but that appreciable quantities remained after DNA replication had virtually

halted (B.E. Wojcik, Ph. D. Thesis, City University of New York, 1988). We wondered whether the residual pools might be located far from replication sites and, hence, be unable to support replication after a shift-up. In particular, we wondered whether there was specific depletion of dNTP pools within the nucleus. Our laboratory has carried out quantitative studies on dNTP pools in rapidly isolated (*ca.* 20 seconds) mammalian cell nuclei (Leeds *et al.*, 1985). The method, described originally by Rapaport *et al.* (1979), involves brief treatment of a cell monolayer with 1% NP-40 detergent, followed by brief washing and then by nucleotide extraction. The dNTP pool sizes in these rapidly isolated nuclei remain stable over at least 2 minutes of extraction (chapter 3) and 30 seconds of washing (Leeds *et al.*, 1985). Since we extract for 10 seconds and wash for five, the nuclear pool sizes that we determine probably represent intranuclear pools as they exist in the intact cell.

When we shifted a JB3-B culture from 33°C to 39°C, we observed an immediate decline in the dNTP pools as measured in whole-cell extracts (Figure 2.1). All four pools declined at similar rates, reaching minimum values by 12 hours after temperature shiftup. The decline roughly paralleled the loss in DNA-synthesizing capacity, as measured by [^{3}H]thymidine incorporation rates

(Dermody *et al.*, 1986). However, substantial residual pools remained at 12 hours, ranging from twenty to fifty percent of the respective pool sizes in the cultures maintained at 33°C. On the other hand, the intranuclear pools declined to virtually undetectable levels. It seems likely, therefore, that the inhibition of DNA synthesis at the non-permissive temperature in JB3-B cells is related to the depletion of DNA precursors within the nucleus.

Is the dNTP pool depletion seen in JB3-B cells directly associated with the *ts* mutation in these cells? To answer this, we analyzed a spontaneous revertant cell line, 3B2R, which was isolated from JB3-B on the basis of normal colony-forming ability at 39°C. The doubling time (28 hours) of the mutant cells was prolonged as compared to the CHO parent (16 hours) but similar to the 3B2R revertant under comparable conditions (B.E. Wojcik, Ph.D. Thesis, City University of New York, 1988). We consequently made comparisons between JB3-B and its revertant rather than with the wild-type parent to ensure that any biochemical difference is due to the mutant gene responsible for the *ts* defect. As shown in Figure 2.2, these cells showed no dNTP depletion after a temperature shift to 39°C, in either whole-cell or nuclear extracts. In fact, most of the pools increased over the 12-hour lifetime of the

experiment, consistent with the idea that the cells continued to grow at 39°C. Therefore, the dNTP pool depletions seen in the JB3-B cells represent a direct consequence of the *ts* mutation in these cells.

In trying to identify the specific enzyme or protein that is affected by the JB3-B mutation, it is instructive to consider the pathways of dNTP biosynthesis. The only enzymes that participate in the synthesis of all four DNA precursors are ribonucleotide reductase and nucleoside diphosphokinase. Since nucleoside diphosphokinase is involved in the synthesis of rNTPs as well as dNTPs, the most direct way to evaluate nucleoside diphosphokinase as the defective gene product was to analyze rNTP pools as a function of time after shiftup of JB3-B cells from 33°C to 39°C. As shown in Figure 2.3, we saw no significant rNTP pool changes in whole-cell extracts under these conditions. Therefore, nucleoside diphosphokinase is ruled out as the mutant protein in JB3-B cells.

Temperature Sensitivity of Ribonucleotide Reductase in JB3-B Cells

Results of the above experiments suggested a critical role for ribonucleotide reductase in mutant and revertant cells. Consequently, we measured the enzyme activity (with CDP as substrate) as a function of time

after shift of cultures from 33°C to 39°C prior to the preparation of enzyme extracts. As shown Figure 2.4, activities in the mutant extracts declined with respect to time after shiftup, with kinetics similar to those that described the dNTP pool depletions. No such loss in activity was seen with the revertant cells, and in fact there was a significant increase in activity during the period analyzed. Thus, the JB3-B mutation affects ribonucleotide reductase activity, either directly or indirectly.

Note, however, that in the mutant cell extracts there were no differences in specific enzyme activity whether the extracts were assayed at 33°C or 39°C. To be sure, the revertant extracts consistently showed increased activity at 39°C as compared with corresponding values obtained at 33°C assay, as one might expect for most wild-type mammalian enzymes. However, the fact that the JB3-B ribonucleotide reductase showed no loss of activity at 39°C suggests that this enzyme might not be the mutant gene product. Another explanation for the data is that the JB3-B ribonucleotide reductase is the thermolabile protein, but that the kinetics of enzyme inactivation are slow with respect to the incubation period in the enzyme assay. We tested this latter possibility by assaying reductase activity, at 33°C, in mutant and revertant extracts, as

a function of time of preincubation of the extracts at either 39°C or 42°C. As seen in Figure 2.5, the JB3-B extracts did show declines in activity after preincubation, while activity in the revertant extracts was unaffected. It should be noted, however, that with the JB3-B extract at 39°C there was a 30-minute lag period before any enzyme inactivation was detectable. Since the incubation period for the ribonucleotide reductase assay is also 30 minutes, it is apparent why the 39°C assay depicted in Figure 2.4 showed no evidence for thermolability of the JB3-B ribonucleotide reductase. More important, the data of Figure 2.5 provide evidence that the JB3-B ribonucleotide reductase protein is thermolabile, implying that the mutation lies in the structural gene for one of the ribonucleotide reductase subunits.

To assess whether the JB3-B mutation directly affects the ribonucleotide reductase gene, similar experiments were performed with the transfectant cells, 206, in which the *ts* defect was corrected with sequences from a human cosmid library (B.E. Wojcik, Ph. D. Thesis, City University of New York, 1988). As shown in Figure 2.6, the transfectant extracts did not show declines in activity after preincubation, consistent with the temperature-independent growth property of the cell line. However, upon Southern blot analy-

sis, the transfectant cells showed no evidence for the acquisition of sequences containing the human ribonucleotide reductase gene (H.L. Ozer, personal communication). Therefore, the possibility of an indirect effect on ribonucleotide reductase activity in JB3-B has not been ruled out.

Discussion

As discussed by Dermody *et al.* (1986) and Ozer *et al.* (1987), relatively few mammalian cell mutants have been described with temperature-sensitive lesions affecting DNA synthesis. The number of these mutants in which defective DNA replication is known to result from aberrant DNA precursor biosynthesis is even smaller; to our knowledge JB3-B is the first one. The observed defect in dNTP biosynthesis correlates closely with the *ts* phenotype. This conclusion is based on several experimental findings. The magnitude and time course of the pool size changes are similar to the inhibition of DNA synthesis measured by [^{3}H]thymidine incorporation (B.E. Wojcik, Ph. D. Theis, City University of New York, 1988). The activity of ribonucleotide reductase, an enzyme responsible for synthesis of all four dNTPs, is diminished in extracts prepared from the mutant at the non-permissive temperature.

Finally, all these properties are restored in a revertant selected for growth at the non-permissive temperature.

Other mammalian cell mutations affecting DNA precursor biosynthesis have been described, but they do not have a conditional lethal phenotype. Mutations affecting ribonucleotide reductase are available (McClarty *et al.*, 1987; Weinberg *et al.*, 1981), but those mutant strains have been isolated on the basis of resistance to hydroxyurea or to deoxyribonucleosides. Others include thymidylate synthase deficiencies that yield a thymidine auxotrophy (Ayusawa *et al.*, 1980), dCMP deaminases deficiencies that cause dNTP pool imbalances (Weinberg *et al.*, 1981), and loss of feedback control of CTP synthetase, which unbalances both rNTP and dNTP pools (Trudel *et al.*, 1984). These cell lines have generated useful insights into biological processes such as mutagenesis (Meuth, 1989; Weinberg *et al.*, 1981), thymineless death (Ayusawa *et al.*, 1983b), and the likely existence of multienzyme complexes for dNTP synthesis (Ayusawa *et al.*, 1983a).

Similarly, we expect the JB3-B mutation to be useful in several ways for further exploration of the relationships between DNA replication and DNA precursor biosynthesis in mammalian cells. For example, we were struck by the fact that estimated DNA synthesis rates

decline in parallel with the depletion of the four dNTP pools, as a function of time after temperature shiftup. Observations from several laboratories have suggested that dNTP levels control rates of DNA replication through interaction with allosteric sites on replicative DNA polymerases (Diffley, 1988; Grindey *et al.*, 1980; Nicander and Reichard, 1981; Reichard, 1978). Such models, we believe, would lead to prediction of more complex relationships between dNTP pool sizes and DNA replication rates than observed in these experiments. Our data must be considered preliminary, however, because we have not yet measured true DNA synthesis rates. Neverthless, the data at this stage suggest a simple relationship between intracellular dNTP concentrations and replication rates, as if polymerase activity were limited after temperature shiftup simply by substrate-level control. Such relationships are suggested also by recent kinetic analyses of DNA polymerase α and of replication in permeabilized mammalian cells (Dresler *et al.*, 1988).

Might one particular dNTP play a dominant role in controlling the rate of DNA replication? A specific role for dCTP has been suggested (Nicander and Reichard, 1981). Our data indicate that the intranuclear levels of all four dNTPs decline in parallel, and they provide no evidence that one of the four deoxyri-

bonucleotides serves a primary regulatory role under these conditions of limited ribonucleotide reductase activity. However, the dNTP contents of our nuclear extracts after temperature shiftup are so low that imprecision is high in our nuclear pool measurements. One way to circumvent this difficulty experimentally could be to attempt to replenish the pool of one or more dNTPs by salvage synthesis from deoxyribonucleosides added to the medium, and then to observe the effects upon DNA synthesis rates. Others who have added deoxyribonucleosides to cultures of hydroxyurea-inhibited cells have reported that salvage-derived dNTPs are not readily used for DNA synthesis (Eriksson *et al.*, 1987; Scott and Forsdyke, 1980; Snyder, 1984). However, in studies of vaccinia virus DNA replication, our laboratory has described conditions under which a hydroxyurea block to ribonucleotide reductase can be bypassed by exogenous nucleosides (M.B. Slabaugh, M.L. Howell, Y. Wang, and C.K. Mathews, submitted for publication).

Before beginning such experiments as described above, it is essential to learn more about the JB3-B mutation. The temperature lability of the enzyme activity in extracts strongly implies a structural mutation affecting ribonucleotide reductase. The relative ease with which temperature-independent revertants

can be isolated suggests that a single mutational event is involved, probably affecting only one of the two subunits. It is important to identify the nature of the mutation, because the metabolic consequences of inhibiting the JB3-B ribonucleotide reductase are distinctly different from the consequences of inhibiting DNA replication in wild-type cells by addition of hydroxyurea. As noted in Figure 2.1, inactivating the JB3-B enzyme by a temperature upshift causes all four dNTP pools to decline in parallel. By contrast, a hydroxyurea block leads to a particularly steep decline in dATP and dGTP pools, while dTTP actually accumulates (Bianchi *et al.*, 1986a). These latter results have been interpreted in terms of rapid turnover of purine dNTP pools, substrate cycles that synthesize pyrimidine dNTPs from nucleosides (Bianchi *et al.*, 1986a), and the ready conversion of intracellular deoxycytidine nucleotides to thymidine nucleotides (Reichard, 1988). Why don't similar consequences ensue from a temperature inactivation of the JB3-B enzyme? Hydroxyurea inhibition is targeted to the small subunit of the tetrameric ribonucleotide reductase, which contains a tyrosine free radical essential to catalytic activity (McClarty *et al.*, 1987; Reichard, 1988). If the JB3-B mutation is found to affect the large subunit, which contains the allosteric control sites for the enzyme, it might

be possible to formulate a model that could account for the differences observed. Does the JB3-B mutation directly affect the structural genes for ribonucleotide reductase? In the 206 temperature-corrected transfectant cells, the ribonucleotide reductase was temperature-stable, but additional DNA sequences for this enzyme were not demonstrated. This suggests the possibility of an indirect effect on ribonucleotide reductase activity being responsible in JB3-B. One possibility is that the mutation affects a protein that interacts with ribonucleotide reductase and stabilizes it.

Finally, we note that even at 30 hours after a temperature upshift, substantial dNTP levels were seen in whole-cell extracts, even though the levels seen in nuclei were nearly undetectable. Does this imply the existence of cytosolic pools of dNTPs, which do not equilibrate with pools in the nucleus? Probably not. Our earlier experiments with synchronized CHO cells showed that under most conditions, dNTP pools in S-phase cells behave as though the cytosol and nucleus constitute a single metabolic compartment (Leeds and Mathews, 1987). However, non-S-phase cells contain substantial dNTP pools, which are obviously not available for use in DNA replication (Leeds and Mathews, 1987). Since the experiments in the present study were

all done with non-synchronized cultures, we suggest that the residual pools seen in whole-cell extracts are confined primarily to cells that are not undergoing DNA replication.

Acknowledgements

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Figure 2.1. Mutant (JB3-B) whole-cell (○) and nuclear (●) dNTP pools after temperature shift from 33°C to 39°C. Cells were seeded at a density of 5×10^5 cells per 100-mm culture dish and incubated at 33°C for 40 hours and then shifted to 39°C at time zero. At the indicated times, whole cells and nuclei were extracted and the dNTP pools were quantitated as described in the text. The pool size is presented as pmol dNTP per 10^6 whole-cells or 10^6 nuclei.

Figure 2.1.

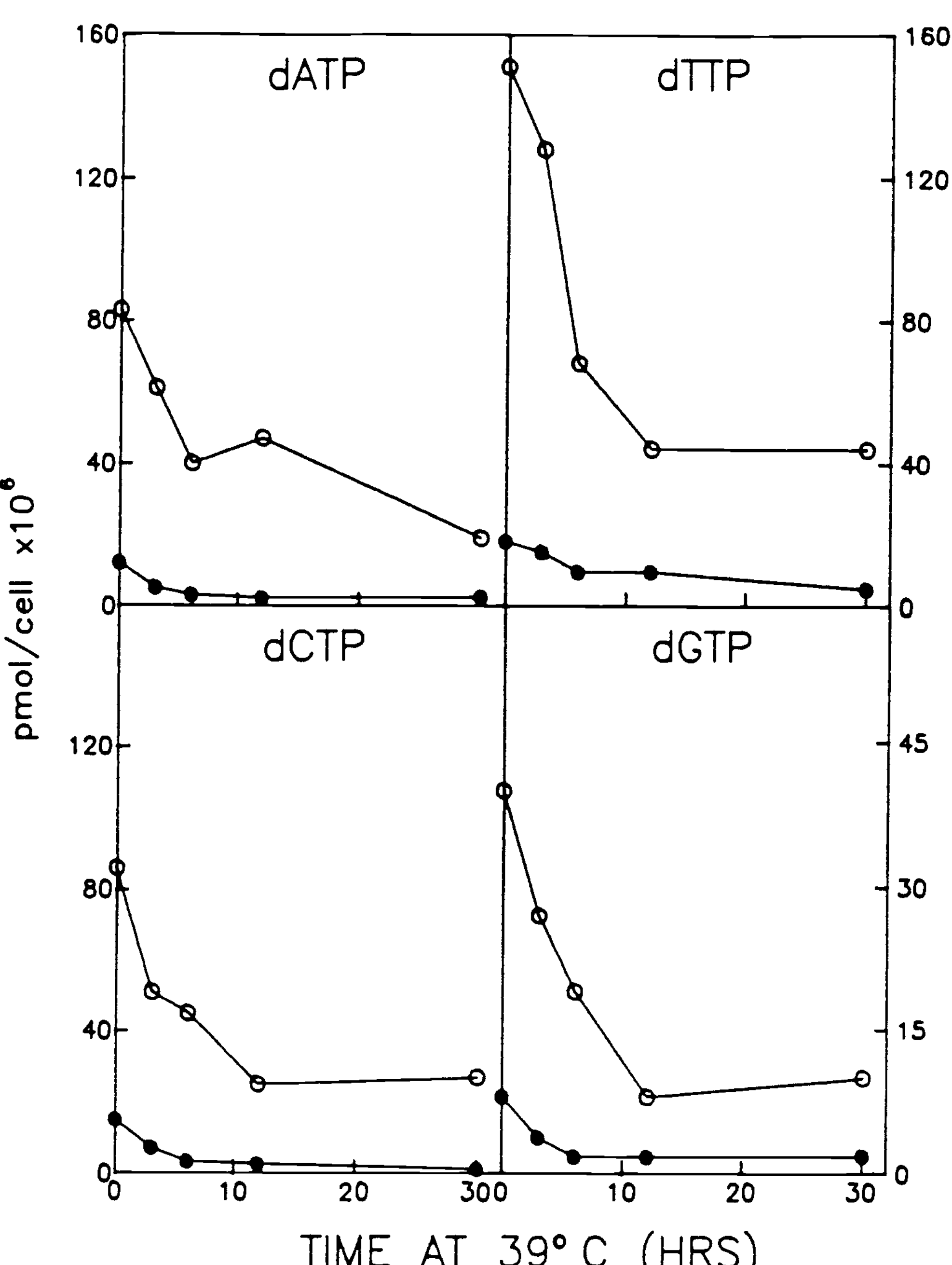


Figure 2.2. Revertant (3B2R) whole-cell (○) and nuclear (●) dNTP pools after temperature shift from 33°C to 39°C. Revertant cells were seeded and incubated at 33°C and then shifted to 39°C identically as described in Figure 1. dNTP pools were extracted and quantitated as described in the legend to Figure 1.

Figure 2.2.

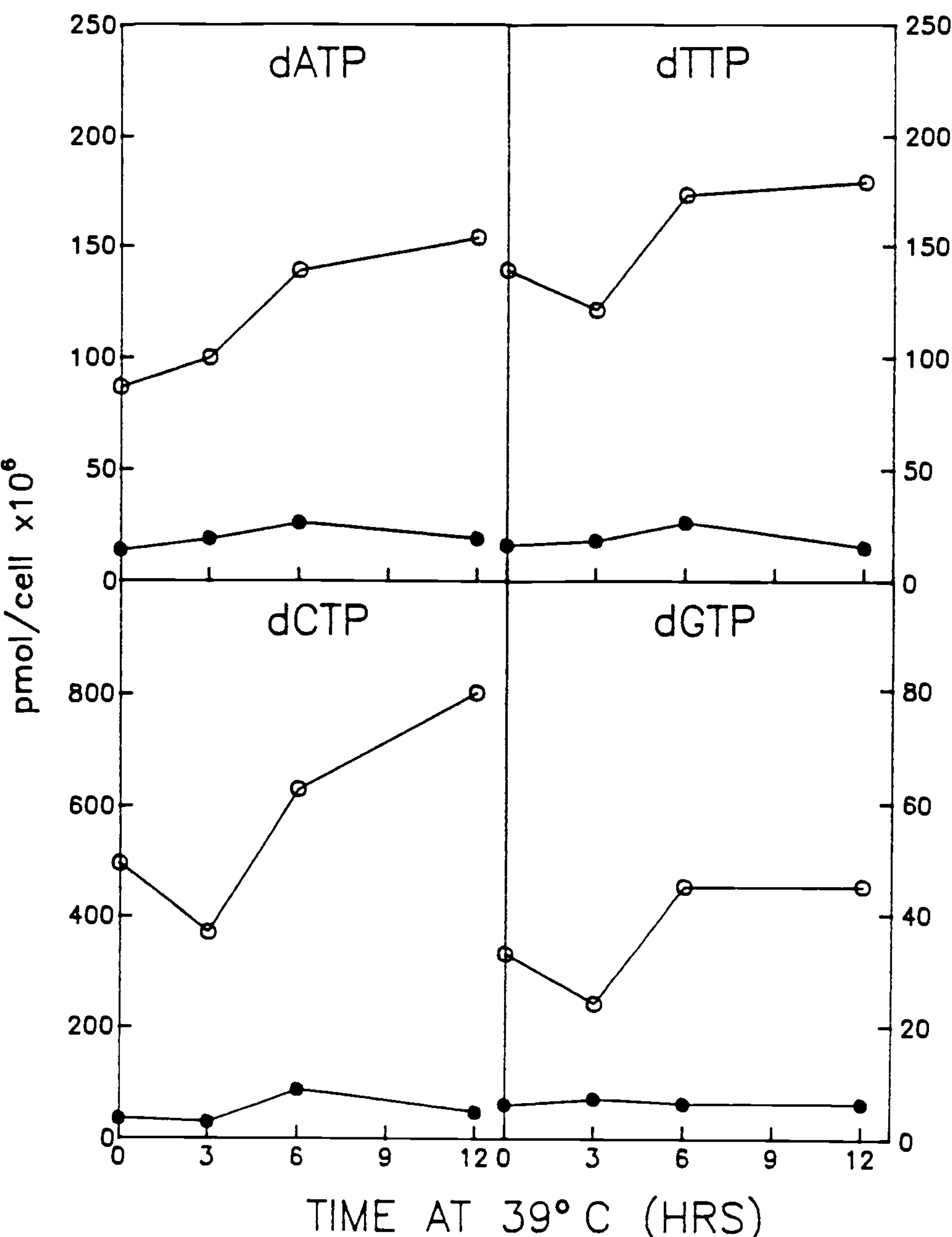


Figure 2.3. Mutant (JB3-B) whole-cell rNTP pools after temperature shift from 33°C to 39°C. The whole-cell extracts used for dNTP pool determination (Figure 1) were quantitated for rNTP content by high performance liquid chromatography as described in the text. The pool size is presented as nmol rNTP per 10^6 whole-cells. Symbols: ATP (■), UTP (★), CTP (▲), GTP (●).

Figure 2.3.

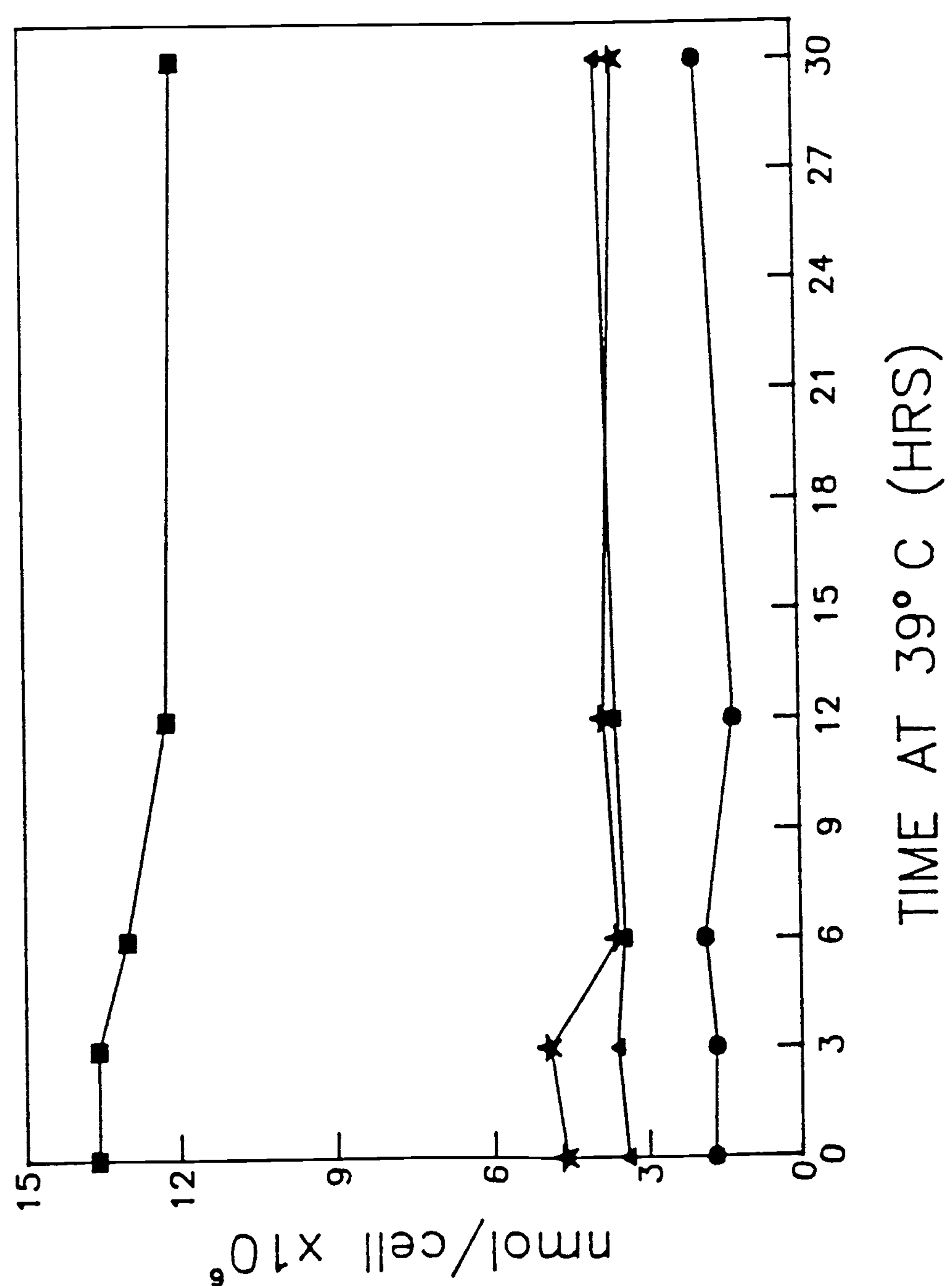


Figure 2.4. Ribonucleotide reductase activity in extracts of mutant (JB3-B) and revertant (3B2R1) cells after temperature shift from 33°C to 39°C. Mutant and revertant cells were seeded at a density of 2×10^6 cells per 100-mm culture dish and incubated at 33°C for 40 hours and then shifted to 39°C at time zero. At the indicated times, the enzyme extracts were prepared and aliquots representing 5×10^6 cells were assayed for CDP reductase activity at 33°C and 39°C as described in the text. Enzyme activity is expressed as picomoles dCDP formed per milligram of protein. Each data point represents the average value of duplicate reductase assays. Symbol: mutant (panel A) assayed at 33°C (●), and at 39°C (▲); revertant (panel B) assayed at 33°C (○), and 39°C (△).

Figure 2.4.

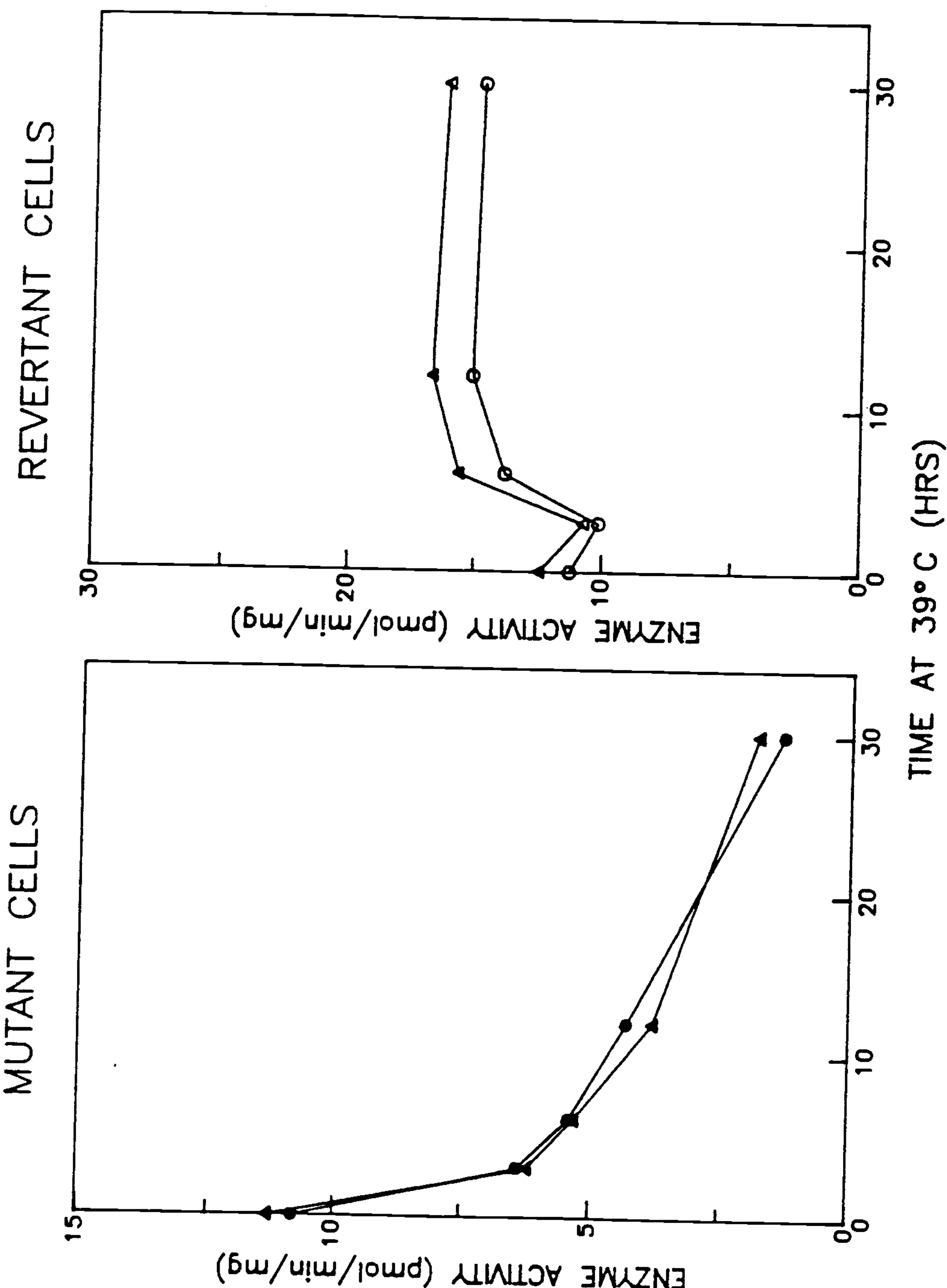


Figure 2.5. Thermolability of ribonucleotide reductase activity from mutant (JB3-B) and revertant (3B2R) cells. The enzyme extracts used for determination of CDP reductase activity (Figure 2.4) were preincubated at 39°C and 42°C for the times indicated in the presence of 40 μM CDP, and then the remaining enzyme activities were assayed at 33°C in duplicate (average value plotted) as described in the text. The activities without preincubation at 39°C or 42°C are designated 100%. Symbols: mutant preincubated at 39°C (●) and 42°C (▲); revertant preincubated at 39°C (○) and 42°C (△).

Figure 2.5.

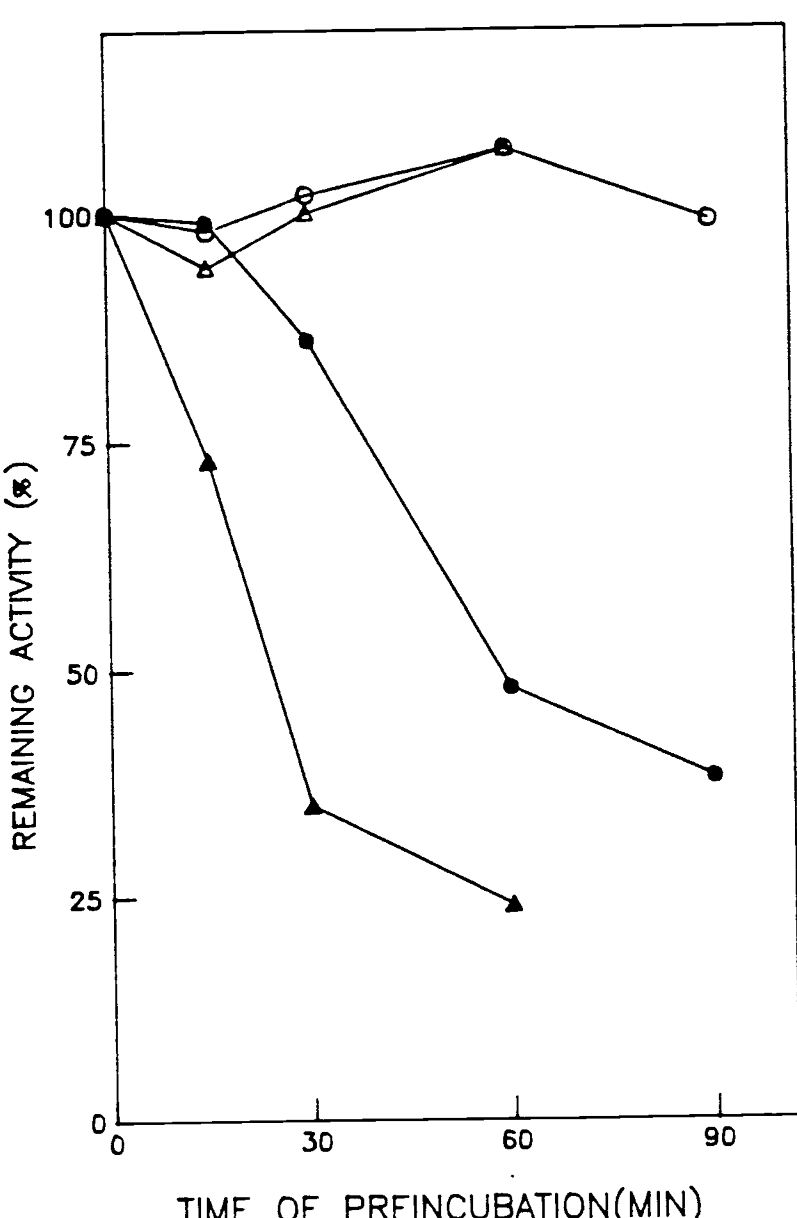
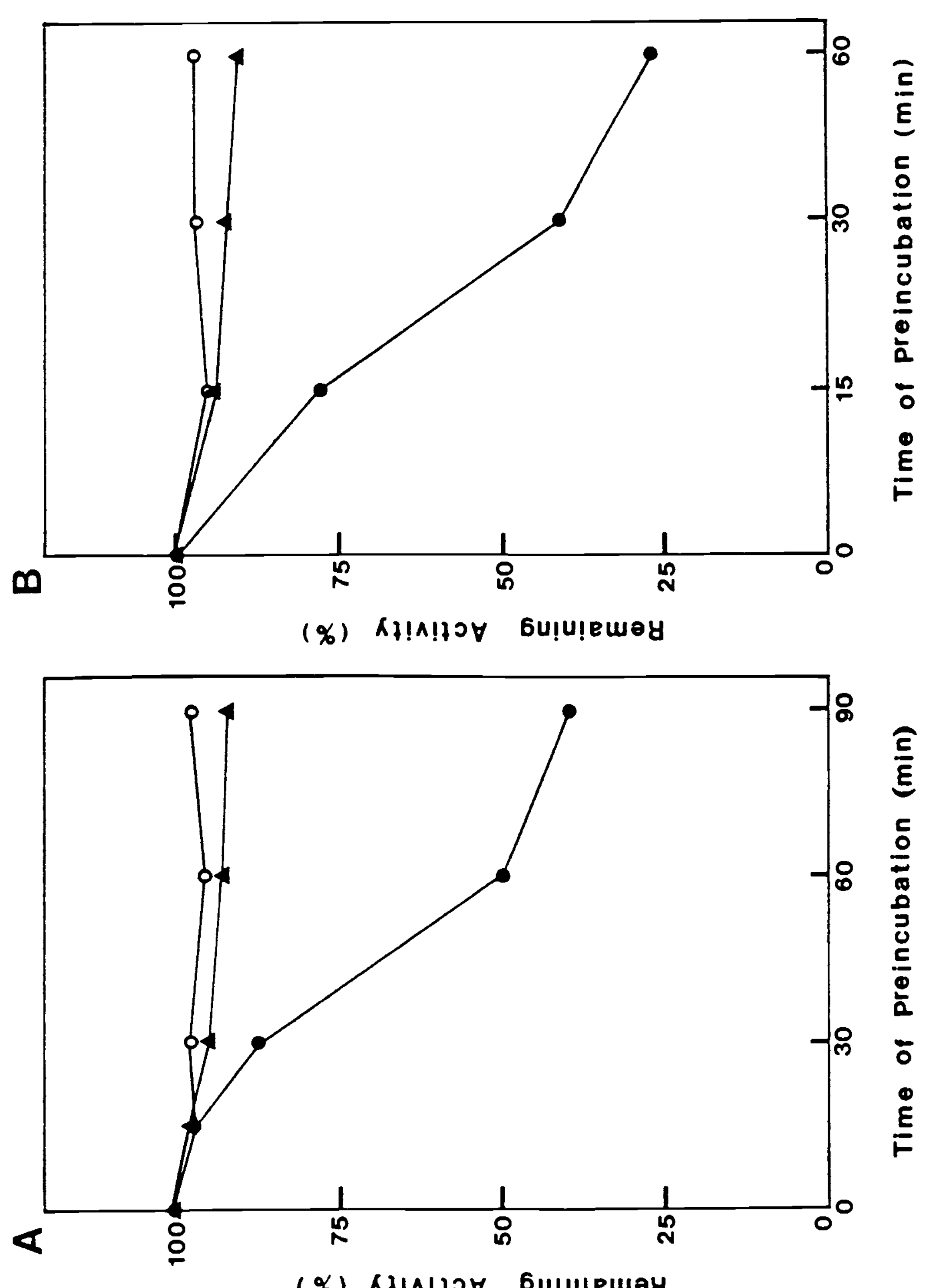


Figure 2.6. Heat sensitivity of ribonucleotide reductase activity from transfectant (206) cells. The experimental protocol was identical to that described in Figure 2.5. Symbols: transfectant (▲) preincubated at 39°C (panel A) and 42°C (panel B). Data for the mutant (●) and revertant (○) were taken from Figure 2.5.

Figure 2.6.



CHAPTER 3

Intranuclear Deoxyribonucleoside Triphosphate Pool Imbalances that are Responsible for the Mutator Phenotype of Thy$^-$ Chinese Hamster Ovary Cells

Byung-Joo Mun and Christopher K. Mathews
Department of Biochemistry and Biophysics
Oregon State University
Corvallis, Oregon 97331

Abstract

Thy$^-$ 49 and Thy$^-$ 303 are Chinese hamster ovary cell mutants that were originally characterized by Meuth (Mol. Cell. Biol. *1*, 652-660 (1981)). Both cell lines have imbalanced deoxyribonucleoside triphosphate (dNTP) pools that result from loss of feedback control of CTP synthetase. While both cell lines have a mutator phenotype, they differ in the extent to which spontaneous mutagenesis is stimulated. We have studied the intranuclear dNTP pool imbalances as the cause of the increased mutation rates in the two mutator lines. When we analyzed the DNA precursor pools from replicating, S-phase Thy$^-$ mutant cells, large imbalances were seen in the extracts of rapidly isolated nuclei while whole-cell extracts showed smaller biases. There was a marked increase in the nuclear dCTP concentrations, apparently resulting from differential nuclear compartmentation in the two strains. A larger dCTP accumulation was seen in Thy$^-$ 303 cells, which have higher mutation frequencies than Thy$^-$ 49. This specific pool increase produced large perturbations in the balance of the nuclear dNTP pools. Thy$^-$ 303 also had more aberrant dNTP pools. The nuclear pool size changes were correlated with the frequencies of mutations observed

in Thy$^-$ mutant cell lines by Meuth: The overall nuclear pool ratio biases were comparable to the increases in mutation frequencies. These data suggest that the intranuclear dNTP pools are determinants of spontaneous mutagenesis in Thy$^-$ mutant cell lines. The results also indicate that dNTPs are compartmentalized in nuclei.

Introduction

The fidelity of DNA replication by various DNA polymerases, in vitro, is strongly affected by deoxyribonucleoside triphosphate (dNTP) pool imbalances (Fersht, 1979; Hibner and Alberts, 1980; Kunkel and Loeb, 1981; Abbotts and Loeb, 1985; Petruska and Goodman, 1985; Kunkel *et al.*, 1986). In cultured mammalian cells induced alterations in dNTP levels, either by exogenous deoxyribonucleosides (Bradley and Sharkey, 1978; Rossman and Stone-Wolff, 1982) or by mutations affecting enzymes of dNTP metabolism (Weinberg *et al.*, 1981; Trudel *et al.*, 1984; Weinberg *et al.*, 1985), have been associated with enhanced mutagenesis. In addition, DNA sequence analysis of mutant regions in a Chinese hamster ovary (CHO) cell mutant has suggested that the increased mutations were due to replication errors induced by the dNTP pool bias (Phear and Meuth, 1989; Meuth, 1989).

However, the relationship between dNTP pool imbalance and the fidelity of DNA synthesis in mammalian cells is often quite obscure. For example, a dCMP deaminase deficiency, which invariably causes dCTP accumulation and dTTP depletion, is not mutagenic in all cell lines (A.L. Arecco and C.K. Mathews, unpub-

lished data). Moreover, the increase in mutation frequency can be uncoupled from the altered pool balance (Caras and Martin, 1988).

Why are enhanced mutagenic effects not always accompanied by dNTP pool size changes in mammalian cells? There is strong evidence for both intra- and inter-cellular compartmentation of dNTPs; the latter indicates that non-replicating cells contain substantial dNTP pools, which are obviously not available for use in DNA replication (Nicander and Reichard, 1983; Nguyen and Sadée, 1986; Duan and Sadée, 1987; Leeds and Mathews, 1987; Spyrou and Reichard, 1987, 1989). Moreover, the sizes of dNTP pools vary considerably throughout the cell cycle (Walters *et al.*, 1973; Skoog *et al.*, 1973; Reichard, 1985), but the levels of nuclear and cytoplasmic dNTPs have been found to vary by different factors in CHO cells (Leeds *et al.*, 1985). Accordingly, the available data based on dNTP pool sizes in whole-cell extracts of non-synchronized cultures will not represent the effective DNA precursor concentrations at replication sites. Thus, these data cannot offer sufficient quantitative explanation of mutagenesis induced by dNTP pool biases in mammalian cells.

In order to fully understand the mutagenic effects of dNTP pool imbalances in cultured mammalian cells,

one must analyze DNA precursor pools in the cell population and in the cell compartment where replication is taking place. In this study, we approached these problems by trying to measure DNA precursor pools in the two major compartments of synchronized, S-phase Thy$^-$ cells, the mutator strains of CHO cells. Investigations of these levels (1) led us to understand the cause of the increased mutations in Thy$^-$ mutator lines, (2) yielded information on the contribution of the intranuclear dNTP pool imbalances to spontaneous mutagenesis, and (3) provided evidence for the compartmentation of dNTPs in nuclei.

Materials and Methods

Materials and Reagents

The following radiolabeled deoxyribonucleoside 5'-triphosphates were obtained from ICN Pharmaceuticals: [8-^{3}H]dATP (22 Ci/mmol), [methyl-^{3}H]dTTP (22 Ci/mmol), [5-^{3}H]dCTP (25 Ci/mmol), and [8-^{3}H]dGTP (11 Ci/mmol). Unlabeled nucleotides and thymidine were obtained from Sigma Chemical Company. The alternating copolymers, poly (dA-dT) and poly (dI-dC) were obtained from Pharmacia, the purified *E. coli* DNA polymerase I (6600 U/mg protein) was purchased from Boehringer-Mannheim, Tri-N-octylamine was obtained from ICN Phar-

maceuticals, Freon-113 (1,1,2-trichlorotrifluoroethane) was obtained from Aldrich Chemical Company, propidium iodide was purchased from Calbiochem-Behring, and Nonidet P-40 (NP-40) was obtained from Particle Delta Laboratories. All other chemicals were of reagent grade.

Cell Lines and Culture Techniques

The wild-type strain and two Thy$^-$ mutants (Meuth *et al.*, 1979) used in these experiments were obtained from Dr. Mark Meuth (Imperial Cancer Research Fund, England). The wild-type strain was the CHO proline-requiring cell line. Thy$^-$ 49 and 303 are independent thymidine-requiring mutants deficient in the allosteric control of CTP synthetase (Trudel *et al.*, 1984). Cells were routinely maintained in monolayer culture at 37°C in Dulbecco's modified Eagle medium (Irvine Scientific) with nonessential amino acids, supplemented with 8% fetal bovine serum (GIBCO) and penicillin-streptomycin (GIBCO). Thymidine (10 μM for wild-type and 100 μM for the Thy$^-$ mutants) was added to all cultures.

For experiments we used dialyzed fetal bovine serum (GIBCO) and defined thymidine concentrations in order to precisely control the exogenous thymidine concentrations.

Isolation of Nuclei

Nuclei were isolated from cell monolayers by the rapid technique developed in this laboratory (Leeds *et al.*, 1985). The method requires less than 20 seconds for the isolation of washed nuclei. Briefly, each 100-mm plate was placed on ice, and the medium was removed by aspiration. The cells were lysed by the addition of 2.5 ml of nuclear isolation buffer (1% NP-40, 30 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 200 mM sucrose, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid]; pH 8.0). The plate was rocked gently for 10 seconds, then the buffer was aspirated and the cells washed for five seconds with 5.0 ml of ice-cold phosphate-buffered saline (PBS). Following aspiration of the PBS, the nuclei remaining on the plate were immediately extracted for determination of dNTP pools. Whole-cell monolayers growing in the same medium were treated identically, except for omission of the nuclear isolation treatment.

dNTP Pool Measurements

Nucleotide extraction and dNTP pool measurements were generally carried out as previously described by this laboratory (North *et al.*, 1980). Both whole cells

and nuclei were extracted by the two-step procedure (North *et al.*, 1980), with two modifications: (1) 5% trichloroacetic acid was used in place of 5% perchloric acid for the second step of the extraction, and (2) acid extracts were neutralized with tri-N-octylamine as described by Garrett and Santi (1979). The contents of three 100-mm culture dishes were pooled for whole-cell assays, and the contents of four dishes were pooled for nuclear assays. dNTP pool sizes were determined by the DNA polymerase-based enzymatic assay (North *et al.*, 1980), which measures incorporation of a limiting dNTP into an alternating copolymer template-primer [poly (dA-dT) or poly (dI-dC)] by DNA polymerase I in the presence of an excess of the labeled complementary dNTP. An additional change was made in the assay procedure: 100 mM dAMP was added to each reaction mixture to limit product breakdown. Aliquots from each assay reaction mixture were taken for measurement at three different times of incubation, to insure that plateau incorporation values were always reached. Also, for each determination two different amounts of extract were assayed, to insure that the radioactivity incorporated was directly proportional to the volume of extract assayed. The values were corrected for both the background incorporation and the sample incorporation for the dilution of the specific activity of the

added radioactive dNTPs by the sample. Results are expressed as pmol dNTP/10^6 whole-cells or 10^6 nuclei.

Cell Synchronization

Cells were synchronized by isoleucine deprivation (Tobey, 1973), using protocols previously described by this laboratory (Leeds *et al.*, 1985). Cells were grown into exponential phase in complete medium plus thymidine at a specified concentration. The medium was aspirated from the cells, and each 100-mm plate was washed twice with 10 ml of PBS. Isoleucine-deficient medium with 5% dialyzed fetal bovine serum and thymidine at the same concentration was added to each plate. Cells were kept in this medium for 30 hours, at which time fresh complete medium was re-added. Cells were harvested by trypsinization at the indicated time points. Aliquots were counted and analyzed by flow cytometry.

Cell Cycle Analysis

Nuclear isolation and DNA fluorochrome staining were combined in a single-step procedure as described by Thornthwaite *et al.* (1980). The cells were left in the nuclear isolation medium (PBS containing 0.6% NP-40, 50 μg/ml of propidium iodide, and 0.2% bovine

serum albumin) for three minutes at room temperature and filtered before analysis. The DNA content of the isolated nuclei was analyzed by measuring the fluorescence of propidium iodide bound to DNA by using an Epics V-cell sorter (Coulter Electronics, Inc.). The proportions of cells in each phase of the cell cycle (G1, S, and G2+M) were obtained by computer-fit analyses of the DNA histograms, by assuming a Gaussian function of the G1 and the G2+M maxima and attributing the remaining part of the DNA histogram to the cells of the S-phase.

Results

The choice of Thy^- mutants of CHO cells was based on observations made in Meuth's laboratory (Meuth, 1981). Two sublines derived from one parental CHO cell line, Thy^- 49 and 303, contain a mutation that interferes with allosteric control of CTP synthetase. Thus, they have elevated dCTP pools and require exogenous thymidine to maintain an adequate dTTP pool for growth (Figure 3.1). Both cells lines also have a mutator phenotype, but the mutation rates were conditional with thymidine concentrations over a range of 1 to 2,000 μM in the medium, a range which varied the dCTP pool and dCTP/dTTP ratio. In 5 μM and 100 μM thymidine, the

difference in mutation rate among three cell lines (between wild-type and Thy^- mutant, and between Thy^- 303 and 49) was maximal and minimal, respectively. However, when grown in 5 μM thymidine rather than 100 μM thymidine, the three cell lines showed smaller differences in the dNTP pools, as measured in non-synchronized whole cells. Why do the smaller differences in the dNTP pools produce a greater difference in mutation rate, either between the two Thy^- mutants or when the mutants were compared to wild-type cells? In attempting to resolve this question, we analyzed nuclear and whole-cell dNTP pools from replicating, S-phase cells grown in 5 μM and 100 μM thymidine. The DNA precursor pool size changes (ratios) were related to the mutation frequencies observed in Thy^- mutant cell lines (Meuth, 1981).

Nuclear and Whole-Cell dNTP Pools in Thy^- Mutator Cell Lines

When grown in medium supplemented with 5 μM thymidine, a growth condition that maximizes differences in mutation frequencies among three cell lines, Thy^- 303 increased its spontaneous mutation rate 140-fold over the wild-type rate, while Thy^- 49 showed an increase of about 15-fold (Meuth, 1981). However, Thy^- 49 and Thy^-

303 have virtually identical dNTP pools, and both mutant lines contain dCTP pools and dCTP/dTTP ratios that are only about five times higher than the wild-type, as measured in extracts of non-synchronized whole cells (Meuth, 1981). Why do Thy$^-$ mutants display such high elevations in mutation frequencies, with relatively small biases in the dNTP pools? Why do the two mutant lines differ in mutation rate by as much as ninefold under very similar pool imbalance conditions? We hypothesized that the Thy$^-$ mutants might differ in ability to compartmentalize dNTPs to the nucleus, such that the effective DNA precursor concentrations at replication sites might vary, with corresponding differences in replicative error frequencies. Thus, we asked whether these differences might result from variations in the intranuclear dNTP pool sizes, rather than in the whole-cell pools. Accordingly, we grew both Thy$^-$ mutant and wild-type cells in a medium containing 5 μM thymidine. dNTP levels were analyzed in extracts of both whole cells and nuclei isolated by a method for the rapid isolation of nuclei (Leeds *et al.*, 1985).

Our laboratory developed a rapid method (*ca.* 20 seconds) for isolating nuclei free of cytoplasmic contamination (Leeds *et al.*, 1985). The method involves a 10-second NP-40 detergent treatment, followed by a 5-second wash. The dNTP values of these rapidly isolated

nuclei were stable over washing intervals of 30 seconds. However, because of the porous nature of the nuclear membrane, we examined the stability of dNTP pools within nuclei isolated by varying the length of treatment with the NP-40-containing nuclear isolation buffer. The sizes of dNTP pools extracted over periods of two minutes of treatment with the nuclear isolation buffer were not reduced (Figure 3.1), confirming negligible leakage of nuclear dNTPs during the isolation procedures. Therefore, it seems likely that the dNTP levels we measured in the nuclear extracts represent true intranuclear pool values.

As shown in Figure 3.3, both mutant lines showed high dCTP pools in the whole-cell extracts, as has been reported by Meuth (1981). Thy^- 49 had a dCTP pool which was approximately five times higher than that of the wild-type. The two Thy^- mutants showed no significant difference in the content of either dNTP, except for a twofold higher dCTP pool in Thy^- 303 as compared with Thy^- 49. The variations in estimates of the dNTP pools obtained in our laboratory could be due to different analytic methods and different proportions of cells in each phase of the cell cycle. The nuclear extracts of Thy^- mutants also showed high dCTP contents, but the expansions over the wild-type dCTP pool were similar to those for the whole-cell extracts. No

other significant differences were observed in the nuclear extracts. This may suggest that the replication forks reflect the pool biases of the total dNTP pools. However, it is unlikely that the small differences in either extract could account for the great differences in mutation frequency. Another explanation for the data is that the dNTP pool sizes estimated in the non-synchronized cultures may differ from the effective DNA precursor concentrations. Hence, they may not fully explain the mutations which occurred through DNA replication errors. To test this possibility, we synchronized the cells and analyzed the intranuclear dNTP pools in the cells of the S-phase, the period of active DNA synthesis.

Cell Cycle Analysis of Synchronized Cells

To compare the DNA synthetic precursor pools of Thy^- mutant and wild-type cells, however, it was necessary to ensure that the Thy^- mutation does not affect cell cycle parameters, so that during experiments we would know that all the cell lines being compared had equivalent proportions of their cells in S-phase under the same conditions of synchronization. Accordingly, we synchronized cultures of both mutant and wild-type cells by 30 hours of isoleucine deprivation, a treatment that readily arrests CHO cells in the G0/G1 bound-

ary (Tobey, 1973) with minimal perturbation of the intracellular dNTP pools (Leeds *et al.*, 1985). The arrested cells were induced to resume cell cycle traverse by the readdition of isoleucine in fresh complete medium. At intervals, the cells were harvested and analyzed by flow cytometry to estimate the proportions of cells in each cell-cycle phase (Figure 3.4). The starting population of asynchronously and exponentially growing Thy$^-$ mutant and wild-type cells (Figure 3.4A) contained about 40% G1 cells, 50% S cells, and 10% G2/M cells. Isoleucine starvation blocked all three cell lines, providing about 90% of the cells in the G1/G0 phase (Figure 3.4B). Upon readdition of isoleucine, the arrested cells resumed the cell cycle traverse, completed G1, replicated their DNA, and divided in synchrony. As shown from the flow cytometric histograms in Figure 3.4, the three cell lines were virtually identical with respect to doubling times under the growth conditions used. The duration of each phase of the cell cycle also appeared to be very similar among the three cell lines. Thus, the pool size differences that might be observed in the synchronized cells should not be a reflection of the different growth rates of the mutant cell lines. Moreover, the addition of 5 μM or 100 μM of thymidine had little effect on the growth rates of the cells (data not shown), although these

additions provided distinct changes in dNTP levels (see below). While others have found that cell cycle parameters are relatively insensitive to change in dNTP pools (Eriksson *et al.*, 1984b), it was necessary to reconfirm this observation for our experiments.

<u>Intranuclear Pool Imbalances in Thy$^-$ Mutator Cell Lines</u>

Mid-S-phase cells in these experiments represented synchronized cultures that had been grown in 5 μM of thymidine, a concentration that maximizes differences in mutation frequency among three cell lines. Under these conditions we saw large pool size changes both in nuclear and whole-cell extracts (Figure 3.5). The expansion of wild-type whole-cell pools was between 1.5 (dATP) and 2.1 (dTTP) times the values observed in the non-synchronized cultures (Figure 3.3). These results appear consistent with the data from exponentially growing whole cells, which contained about 50% S-phase cells. Both mutant cell lines elevated dCTP pools, as expected. However, these whole-cell pool variations during the S-phase did not produce significantly larger differences among the three cell lines than observed for the random-phase cultures. However, an unexpected finding was that the dGTP pool is strikingly lower in Thy$^-$ 303 cells than either wild-type or Thy$^-$ 49 cells.

The nuclear pools of DNA precursors varied differently during the S-phase, changing the relative nuclear dNTP pool ratios. The two mutator cell lines showed marked increases in the intranuclear dCTP pools when compared with the parental cells. Extreme pool biases were observed for the nuclei of Thy^- 303, which has a more pronounced mutator phenotype than Thy^- 49. There was a larger elevation in the dCTP pool. Moreover, the dGTP pool showed a reduction. Certainly, these nuclear pool measurements revealed dramatic differences among the three cell lines that were not observed in the analyses of the whole-cell extracts. Thy^- 303 had dCTP pool and dCTP/dTTP ratios which were, respectively, 42- and 57-fold higher than the wild-type, whereas Thy^- 49 had, respectively, 13- and 17-fold higher corresponding values, displaying differences which were, respectively, 3.2 and 3.4 times the parameters between the two mutator cell lines. Therefore, it is likely that the <u>nuclear</u> pool imbalance is more directly related to the increased mutations in Thy^- mutant cell lines than is the imbalance in whole-cell pools. The fact that great differences in the nuclear pools were seen among the three cell lines, despite small differences in the whole-cell pools, suggests that some factor other than simple diffusion may distribute intracellular dNTPs across the nuclear membrane; otherwise, the difference

in pool sizes would be the same in both extracts. We calculated the nuclear pool as a percentage of the whole-cell pool (Table 3.1), based upon the above data.

Differential Nuclear Compartmentation of the dCTP Pool in Thy$^-$ Mutator Cell Lines

As shown in Table 3.1, a higher fraction of the total purine dNTP content was retained in the nucleus than the nuclear fraction of pyrimidine dNTPs in the non-synchronized cultures of both mutant and wild-type cells, suggesting a non-random distribution of dNTPs between the two cellular compartments. During S-phase, the four dNTPs appeared to be equilibrated in the wild-type cells. The relatively low nuclear pool sizes seen in the S-phase would be due to their rapid turnover for nuclear DNA replication, although a major requirement for dNTPs exists within the nucleus during that period. However, Thy$^-$ mutants showed an abnormally large accumulation of the dCTP pool in the nucleus during the S-phase, with a larger expansion observed for Thy$^-$ 303. The other three dNTPs were localized in the nucleus at percentages similar to the corresponding wild-type proportions. The fact that pool imbalance was more pronounced in the nuclear extracts, plus the fact that a specific increase was seen in the nuclear dCTP pool

during the S-phase, indicates that the differential nuclear compartmentation of the dCTP pool is a direct cause of the increased mutations in the Thy$^-$ mutants. This specific pool size change, then, should be related to the types and frequencies of mutations induced in the Thy$^-$ mutant cell lines.

Intranuclear Pool Ratio Perturbations Created by the Increased Nuclear dCTP Pool in Thy$^-$ Mutator Cell Lines

Since misincorporation of the nucleotide in excess was found to be the major mutation pathway in the adenine phosphoribosyl-transferase (aprt) gene of Thy$^-$ mutant cells (Phear *et al.*, 1987; Phear and Meuth, 1989), competition between correct and incorrect nucleotides at insertion sites should be the main factor governing the frequencies of the mutations. The ratio of incorrect and correct nucleotides should, then, affect the frequency of the mutations. Each ratio should influence the corresponding mutation frequency, and the overall pool ratio perturbations should be correlated with the change in total mutation frequencies. Therefore, it may be reasonable to expect that the accumulative effects are additive, while the quantitative relationship between dNTP pool ratio and mutagenesis has yet to be defined due to the frequently

involved DNA sequence context effect (Fersht, 1979). To test this, we calculated the pool ratios for each extract (Table 3.2), based on the data presented in Figure 3.5.

As we expected from the specific nuclear dCTP pool increase in Thy^- mutants, the only significant perturbations seen were in the ratios of [dCTP]/[dATP], [dCTP]/[dTTP], and [dCTP]/[dGTP]. While we observed some differences in the whole-cell extracts, the nuclear extracts displayed much larger biases in the three pool ratios; these more readily account for the large differences in mutation frequencies. Thy^- 303 nuclei displayed [dCTP]/[dATP], [dCTP]/[dTTP], and [dCTP]/[dGTP] pool ratios that were, respectively, 73, 57 and 78 times higher than the corresponding values for the wild-type, while the Thy^- 49 nuclei showed increases that were, respectively, 18, 16 and 12 times higher, producing 4-, 3- and 6-fold differences in these parameters between the two Thy^- mutants. Moreover, the overall perturbations in these three nuclear pool ratios were comparable to the changes in mutation rates of the Thy^- mutator cell lines. For example, if the 4-, 3-, and 6-fold differences were additive, then the differences could serve to explain the 9-fold difference in mutation frequencies between Thy^- 303 and 49, when it is considered that some of the misincorpo-

rated nucleotides for the mutations, which show the next nucleotide effect, would be removed by editing functions. The same was observed between Thy$^-$ mutant and wild-type cells when the difference in nuclear pool ratio was compared to the difference in mutation frequency.

Are the intranuclear pool size differences among the three cell lines actually related to the differences in mutation frequencies? If so, then we might expect to see the nuclear pool size differences diminish or disappear under growth conditions where the differences in mutation frequency are small. Meuth (1981) observed that the two mutant cell lines differed very little in spontaneous mutation frequency, and Thy$^-$ mutant and wild-type showed only small differences in mutation rates when grown in a medium containing 100 μM thymidine. Accordingly, we grew and synchronized cells in 100 μM thymidine. As shown in Figure 3.6, the whole-cell extracts showed some differences. However, the nuclear pools were nearly identical for Thy$^-$ 49 and Thy$^-$ 303, and the differences in nuclear extracts between Thy$^-$ mutant and the wild-type were not significant. Thus, it is apparent that intranuclear pool size changes are related to the increased mutation rates in the Thy$^-$ mutator cell lines. Accordingly, our results suggest that the dNTP pool sizes at the replication

fork are reflected more accurately in the dNTP pools measured in the nuclear extracts than in the whole-cell pools. The results also indicate the existence of nuclear compartmentation of dNTPs.

Discussion

This study shows the importance of determining dNTP pool sizes in the nucleus of replicating, S-phase cells, which represent the effective concentrations of DNA synthetic precursors at replication sites, in order to fully understand the mutagenic effects of dNTP pool imbalances in cultured mammalian cells. While dNTP pool sizes measured in non-synchronized whole cells did not explain the higher rates of mutations in the Thy^- mutant cell lines, the nuclear pool size changes in the S-phase cells can clearly be used to explain the mutation rates.

Several experimental findings have shown that the intranuclear dNTP pool imbalances are the cause of the mutator activity in the Thy^- mutant cell lines: (1) The extracts of rapidly isolated nuclei showed large imbalances, which clearly account for the hypermutability of the Thy^- mutants, while smaller biases were observed in the whole-cell extracts. Extreme pool biases were observed in the nuclei of Thy^- 303, which

has a more pronounced mutator phenotype than Thy$^-$ 49. (2) The differences in the nuclear dNTP pools among the three cell lines (wild-type and the two Thy$^-$ mutants) were related to the differences in mutation rates. Under conditions for maximized and minimized differences in mutation rates, we observed differences in the nuclear dNTP pools which were, respectively, large and small. (3) The correlation of nuclear pool size changes with the frequencies of mutations observed in the Thy$^-$ mutant cell lines (Meuth, 1981) seems to have been good. There was a specific increase in the nuclear dCTP pools. The overall nuclear pool ratio biases created by the increased dCTP pool were comparable to the increase in mutation frequencies in the Thy$^-$ mutants when the DNA sequence context effect was considered. Therefore, the intranuclear dNTP pools are a more predictive determinant of spontaneous mutagenesis in Thy$^-$ mutator cell lines.

Since the increased mutational rates observed in Thy$^-$ mutants were conditional with thymidine concentrations, which manipulated the dCTP pool and [dCTP]/[dTTP] ratio, Meuth (1981) suggested that the changes in the dCTP and dTTP pools were related to the mutation frequencies induced in Thy$^-$ mutant cells. Do only the pyrimidine dNTP pool size changes influence the frequencies of mutations in the Thy$^-$ mutator cell lines?

If so, the perturbation would be seen only in the [dCTP]/[dTTP] ratio, which stimulates T → C transition, since mutagenesis induced by dNTP pool imbalance probably proceeds largely via competition for incorporation between correct and incorrect dNTPs (Phear *et al.*, 1987; Phear and Meuth, 1989). However, all three ratios, [dCTP]/[dATP], [dCTP]/[dTTP] and [dCTP]/[dGTP], show large biases for both Thy$^-$ 303 and Thy$^-$ 49. Moreover, the overall perturbations in these three pool ratios are comparable to the increases in mutation in the Thy$^-$ mutant lines, while the [dCTP]/[dTTP] ratio bias alone is not large enough to account for the higher mutation rates. The fact that three types of base substitutions (A → C, T → C, and G → C mutations) were identified in the Thy$^-$-induced aprt$^-$ mutants (Phear *et al.*, 1987; Phear and Meuth, 1989), i.e., the T → C transition was not only the mutation type, is consistent with our observations. Therefore, the relative sizes of all four dNTP pools seem to contribute toward mutation frequencies. It seems unlikely that only the pyrimidine nucleotide pool imbalances will affect the spontaneous mutation rates.

Another conclusion from these experiments is that dNTPs are compartmentalized in the nuclei. The differential nuclear compartmentation of the dCTP pool produced larger imbalances in the nuclear than the whole-

cell dNTP pools, resulting in mutator activity in the Thy$^-$ mutant cell lines. This finding, plus the fact that the intranuclear dNTP pools were the determinants of spontaneous mutagenesis, as described above, clearly indicates the existence of nuclear compartmentation of dNTP pools.

How, then, can dNTPs be present at different concentrations in the two cellular compartments with no apparent permeation barrier for the nucleotide? During the cell cycle the ratio of nuclear to whole-cell dNTP varied: There was a distinctive distribution of purine and pyrimidine dNTPs between nucleus and cytoplasm in the non-synchronized cultures, while there was a symmetric distribution during the S-phase. In addition, the Thy$^-$ mutant lines showed a cell cycle-dependent expansion in nuclear dCTP proportions. These data suggest that the intracellular distribution of dNTPs is controlled by a cell cycle-dependent regulation.

Leeds and Mathews (1987) demonstrated that dCTP pools were equilibrated to equivalent specific activities within CHO cells after incubation of the cells with radiolabeled nucleoside, both in S and G1 phases. In addition, in the present study Thy$^-$ mutants showed some perturbations in whole-cell pools, even though the biases were much smaller than those observed for the nuclear extracts. These observations suggest that

dNTPs are freely permeable across the nuclear membrane. However, the large nuclear accumulation of dCTP pools in the Thy^- mutant lines suggests an essentially unidirectional flow of dNTPs from the cytoplasm into the nucleus, rather than a random diffusion. In support of this concept, our data show that the nuclear dNTP pool sizes more accurately reflect the effective dNTP concentrations at replication sites, hence acting as determinants of spontaneous mutagenesis.

If the four dNTPs can be equilibrated within the cell during S and G1 phases, as suggested by the data, the asymmetries in the nuclear to whole-cell pool ratios observed in the non-synchronized cultures would be due to the G2/M phase cells. If so, what prevents a nuclear accumulation of pyrimidine dNTPs during the G2/M phases? From our results, we hypothesize that pyrimidine nucleotide-catabolizing activities may exist in the nucleus after DNA synthesis (G2/M phases), thereby producing subtle changes in the nuclear dNTP proportions during the cell cycle (i.e., before and after DNA synthesis). Nicander and Reichard (1985b) have proposed that the enzyme(s) catalyzing pyrimidine dNTP turnover increase in activity when cells do not need high levels of dNTPs by the inhibition of DNA replication.

While both Thy$^-$ 49 and Thy$^-$ 303 are derived from one parental CHO cell, and both have a similar phenotype, they display differences in the dNTP pool sizes and thereby differences in mutation frequencies. Both cell lines contain elevated dCTP pools, but Thy$^-$ 303 cells show a higher dCTP pool, both in nuclear and whole-cell extracts. In addition, the dGTP pool is lower in Thy$^-$ 303 cells than in Thy$^-$ 49 cells. What could be the basis for the differences between these two mutant cell lines? As shown earlier by Meuth and his colleagues (Trudel *et al.*, 1984), both cell lines carry a mutation that abolishes feedback control CTP synthetase; this explains the vastly elevated dCTP pools in both cell lines. Moreover, differential nuclear compartmentation of dCTP pools observed in the present study accounts for the larger dCTP accumulation in the nuclei of Thy$^-$ 303 cells. However, our data suggest that Thy$^-$ 303 cells contain at least one additional mutation, which could be responsible for the unusually low dGTP pool in these cells.

What might be the nature of this putative second mutation? Note that the size of the dCTP pool is larger in Thy$^-$ 303 than in Thy$^-$ 49 cells. The only enzyme responsible for the synthesis of both dCTP and dGTP is ribonucleotide reductase, and allosteric control of this enzyme in vivo is known to affect the pool

sizes of all four dNTPs (Thelander and Reichard, 1979; Reichard, 1987). Moreover, the activity of this enzyme is highly cell cycle-dependent. Thus, our results suggest one possible explanation: that Thy$^-$ 303 cells bear an additional mutation affecting the specificity site of ribonucleotide reductase large subunits, such that binding of dTTP to the specificity site were diminished. Decreased binding of dTTP would simultaneously activate CDP reduction and inhibit GDP reduction, thereby increasing the dCTP pool and decreasing the dGTP pool. However, when Trudel *et al.* (1984) tested the sensitivity of dTTP for CDP reduction in non-synchronized cells, a small difference was observed between Thy$^-$ 49 and Thy$^-$ 303. These results are consistent with our data for dNTP pool sizes measured in non-synchronized cells. However, note that expression of the second mutant phenotype is apparently cell-cycle-dependent, since reductions in the dGTP pool are detectable only in the S-phase-enriched cell population. Therefore, our experimental results could be explained by a second mutation, whose phenotype is observed only at a certain phase of the cell cycle. This model has the advantage of being easily tested: The inhibitory effects of dTTP on CDP reduction in extracts synchronized S-phase cells could be analyzed. Such experiments are currently in progress.

Acknowledgement

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Figure 3.1 Metabolic alterations in Thy^- mutants. CHO cells contain little or no dCMP deaminase activity. The Thy^- mutation diminishes sensitivity of CTP synthetase to feedback inhibition by CTP. Thus, the Thy^- mutant cells exhibit increased flux through the CTP synthetase reaction. Reactions whose flux rates are increased in Thy^- cells are denoted with heavy arrows, and reactions with decreased flux rates are shown with dashed arrows.

Figure 3.1.

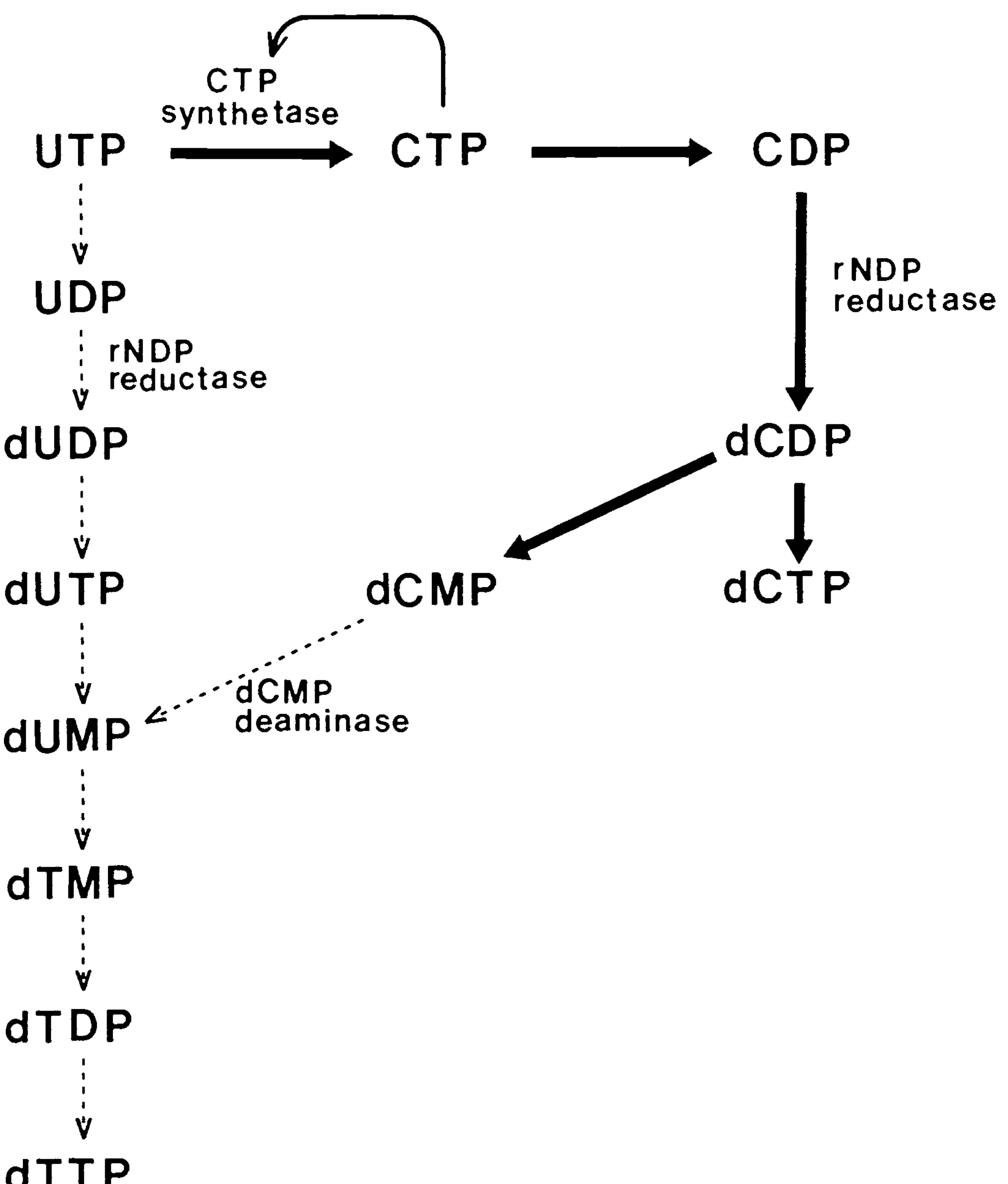


Figure 3.2. Nuclear dNTP pools versus time of treatment with NP-40-containing nuclear isolation buffer. Exponentially growing wild-type cells in 5 μM thymidine were treated with the NP-40-containing buffer for 10, 30, 60, 90, or 120 seconds, and the nuclear dNTPs were then extracted and quantitated. The pool size is presented as pmol dNTP per 10^6 nuclei.

Figure 3.2.

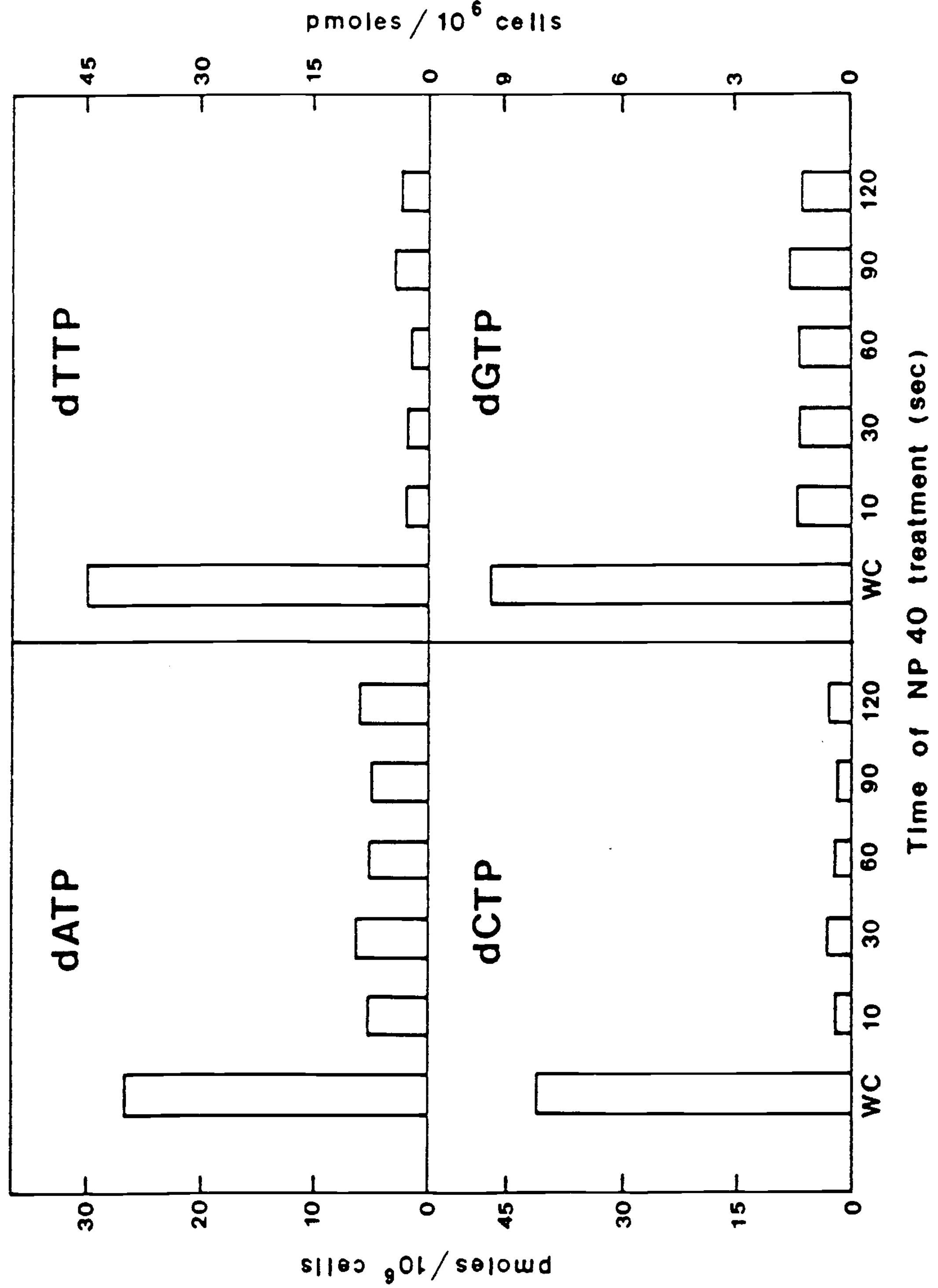


Figure 3.3. Nuclear and whole-cell dNTP pools from non-synchronized wild-type and Thy$^-$ mutant cells grown in 5 μM thymidine. Cultures growing exponentially in medium containing 5 μM thymidine and dialyzed serum were harvested, and the dNTP pools were extracted and quantitated both from rapidly isolated nuclei and from cells. The thymidine concentration was controlled three days prior to harvest and analysis of the pool content. The pool size is presented as pmol dNTP per 10^6 whole-cells or 10^6 nuclei. Results shown here represent averaged values from two identical experiments. The two experiments agreed within 10% of each other. (☐), wild-type; (▨), Thy$^-$ 49; (▤), Thy$^-$ 303.

Figure 3.3.

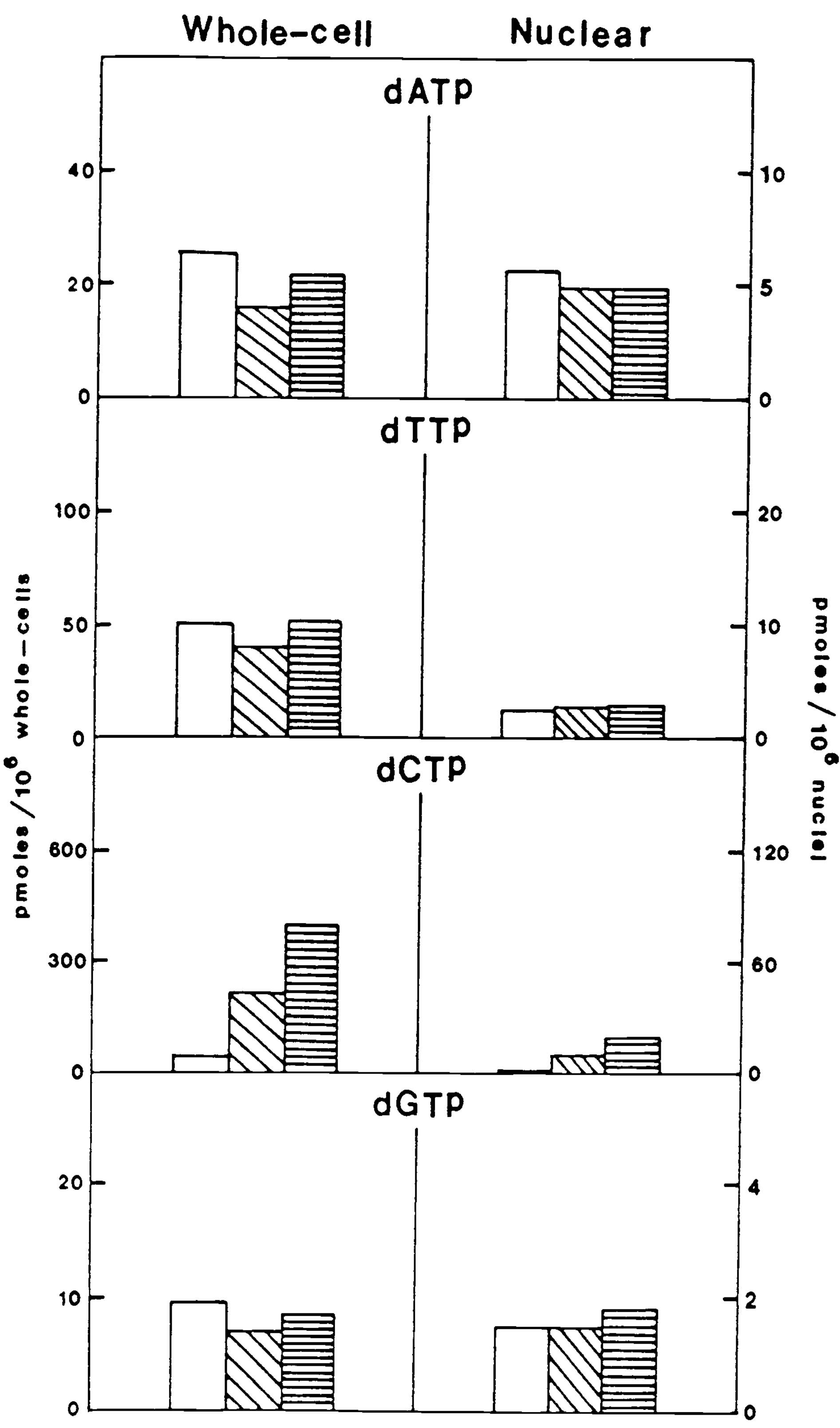


Figure 3.4. DNA distribution of synchronized wild-type and Thy$^-$ mutant cells. Exponentially growing cells in 5 μM thymidine were arrested in the G0/G1 phase of the cell cycle by 30 hours of isoleucine deprivation, and the cells were then allowed to traverse the cell cycle by readdition of isoleucine. At the indicated times, nuclei were prepared from the cells and the DNA contents were analyzed by flow cytometry: (A) DNA distribution before isoleucine starvation; (B) DNA distribution, showing cells predominantly in the G1 phase, after 30 hours of isoleucine deprivation; (C) DNA distribution, showing most cells in the S-phase at 12 hours after isoleucine readdition; (D) DNA distribution, showing most cells undergoing mitosis at 15 hours after isoleucine readdition.

Figure 3.4.

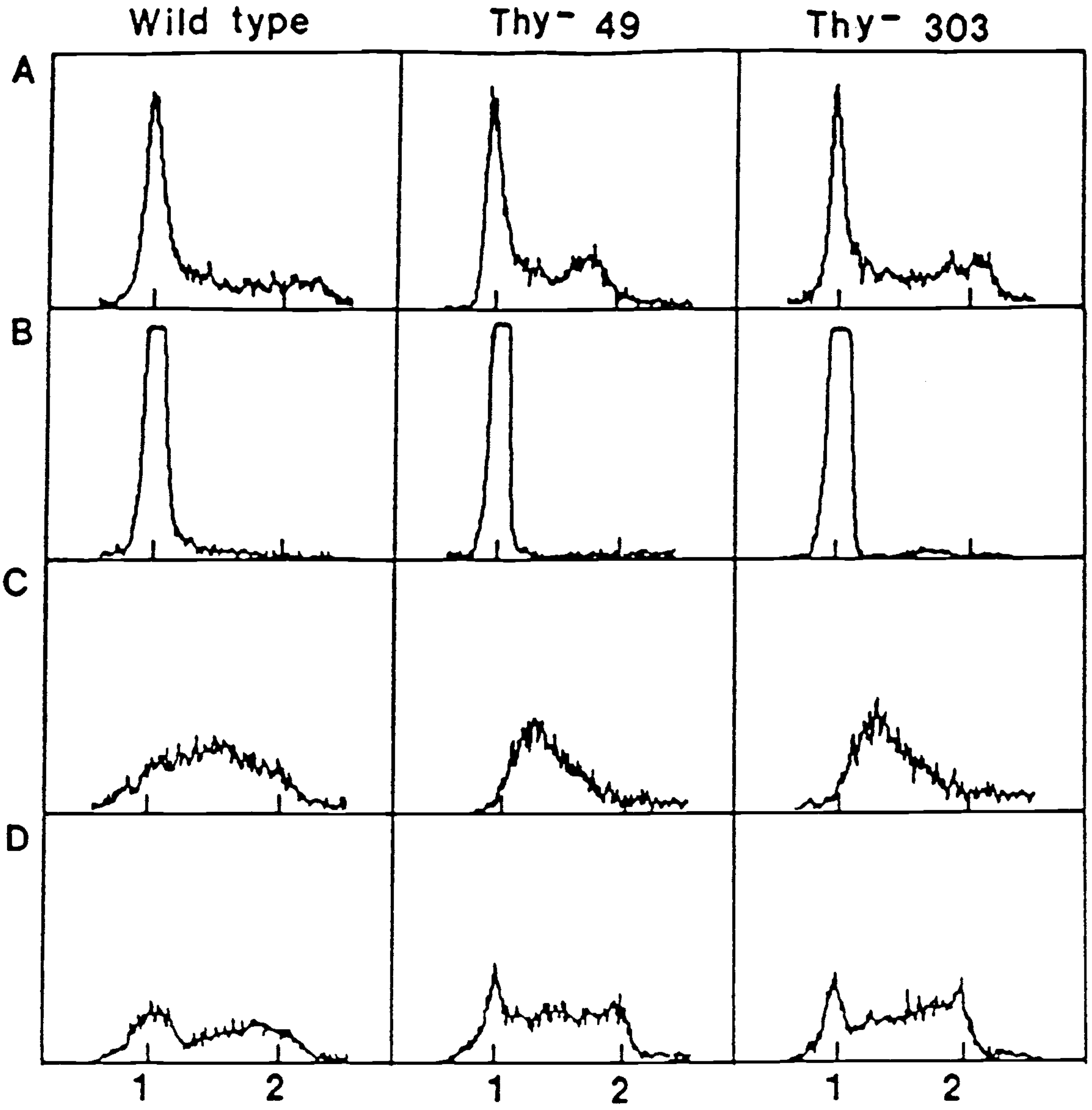


Figure 3.5. Nuclear and whole-cell dNTP pools from synchronized, S-phase wild-type and Thy$^-$ mutant cells grown in 5 μM thymidine. Cultures in exponential growth were synchronized by 30 hours of isoleucine deprivation in medium containing 5 μM thymidine and dialyzed serum. The thymidine concentration was controlled two days prior to synchronization. At 12 hours after isoleucine readdition, the dNTP pools were extracted and quantitated from both rapidly isolated nuclei and from cells. Results shown here represent averaged values obtained in two identical synchronizations. The two experiments agreed within 10% of each other. (open box), wild-type; (diagonally hatched box), Thy$^-$ 49; (horizontally hatched box), Thy$^-$ 303.

Figure 3.5.

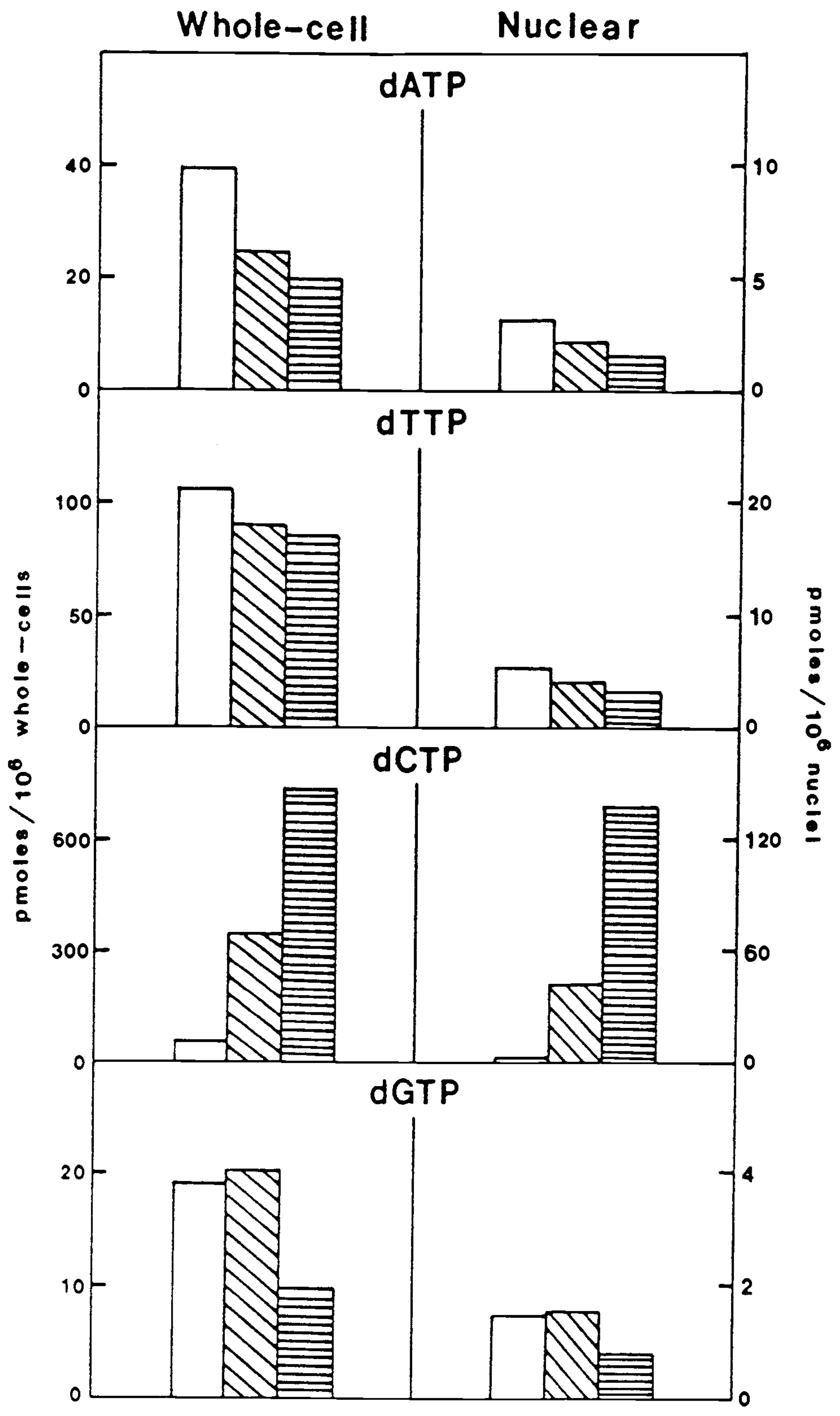


Table 3.1. Proportion of nuclear pool to the whole-cell dNTP pool in S-phase cells and non-synchronized cultures.

	Nuclear pool as % of whole-cell pool[a]			
Cell line	dATP	dTTP	dCTP	dGTP
		S-phase		
Wild-type	7.7	4.9	5.9	7.8
Thy^- 49	8.4	4.4	12.3	7.5
Thy^- 303	8.8	4.5	18.6	8.3
		Non-Synchronized		
Wild-type	21.5	5.0	4.6	15.0
Thy^- 49	30.1	6.8	4.4	21.0
Thy^- 303	20.9	4.9	4.7	20.6

The data used for determination of these percentages are those presented in Figures 3.3 and 3.5.

[a] Each whole-cell dNTP is designated 100%.

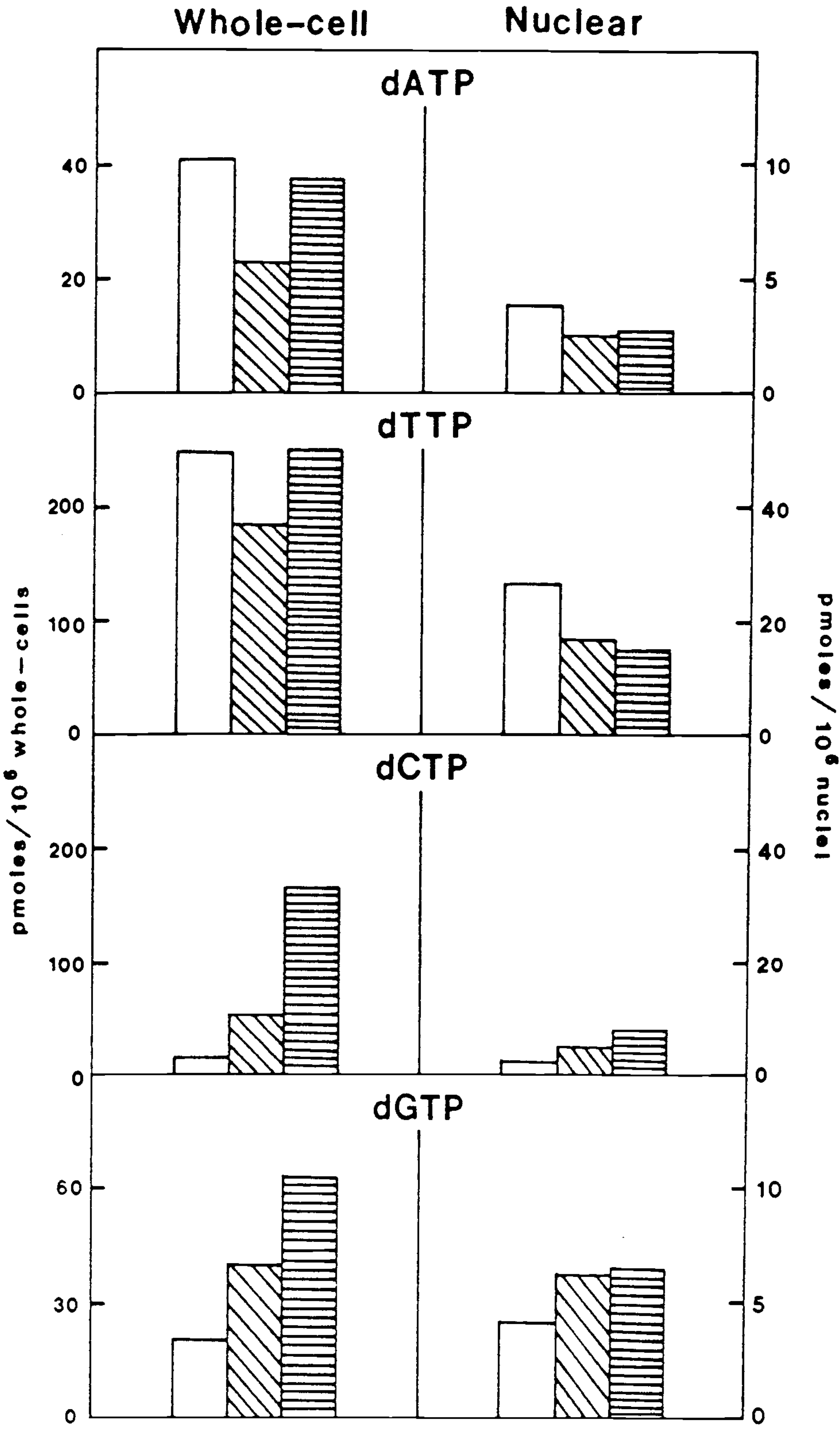
Table 3.2. Perturbations of nuclear and whole-cell pool ratios in S-phase cells grown in 5 μM thymidine.

Cell line	dCTP/ dATP	dCTP/ dTTP	dCTP/ dCTP	dATP/ dGTP	dTTP/ dGTP	dTTP/ dATP
			Nuclear			
Wild-type	1.1	0.6	2.2	2.0	3.5	1.7
Thy^- 49	20.2	10.6	28.3	1.4	2.7	1.9
Thy^- 303	80.6	36.1	171.3	2.1	4.8	2.2
			Whole-Cell			
Wild-type	1.4	0.5	2.9	2.0	5.6	2.7
Thy^- 49	13.9	3.8	17.3	1.3	4.5	3.6
Thy^- 303	38.0	8.7	76.8	2.0	8.8	4.4

The data used for determination of these ratios are those presented in Figure 3.5.

Figure 3.6. Nuclear and whole-cell dNTP pools from S-phase wild-type and Thy^- mutant cells grown in 100 μM thymidine. The experimental protocol was identical to that described in Figure 3.5, except that the thymidine concentration was 100 μM throughout. Results shown here represent averaged values obtained from duplicate cultures. The duplicate assays agreed within 5% of each other. (□), wild-type; (▨), Thy^- 49; (▤), Thy^- 303.

Figure 3.6.



CHAPTER 4

Discussion

In an effort to understand the extent to which rate and fidelity of DNA replication are regulated directly through the availability of its precursors, two distinct mammalian cell mutants, which are defective in deoxyribonucleotide metabolism, have been analyzed. (1) I investigated a temperature-sensitive DNA^- mutant of CHO cells, showing it to be defective in ribonucleotide reductase activity, and studied the dependence of DNA replication rate on precursor concentrations. (2) I analyzed two mutator strains of CHO cells, deficient in control of CTP synthetase, investigating the dependence of DNA replication fidelity on the balance of DNA precursors. Since DNA synthesis obviously occurs in the nucleus, it was of particular interest to explore the regulatory effects of intranuclear dNTP pools on DNA replication.

Intranuclear dNTP Pools as Determinants of DNA Replication Rate

To define the control of DNA replication rate by dNTP levels, the phenotype of a CHO cell mutant, which is temperature-sensitive for DNA replication, was characterized. Inactivation of a thermolabile form of ribonucleotide reductase activity by temperature upshift of a mutant culture diminished dNTP pools and thereby halted DNA synthesis. All four dNTP pools declined at similar rates in extracts of both rapidly isolated nuclei and whole cells, as a function of time after shift of a mutant culture to the non-permissive temperature. The magnitude and time course of the pool size changes measured in both extracts were parallel with the inhibition of DNA synthesis, measured by [^{3}H]thymidine incorporation. However, the estimated DNA synthesis rate was more closely related to the depletion of DNA precursors within the nucleus. When intranuclear dNTP pools were completely depleted, at about the same rate as DNA synthesis was shut off, whole-cell pools were drained, but not completely.

The data suggest (1) that the activity of ribonucleotide reductase determines the sizes of the four dNTPs, (2) that the availability of the four dNTPs influences the DNA replication rate to a certain

extent, but (3) that the intranuclear concentrations of DNA precursors more closely correspond to the rate of DNA synthesis than those in whole-cell extracts, indicating the possibility of nuclear compartmentation of dNTPs. These conclusions, in turn, suggest a simple, first-order relationship between dNTP pool size and DNA replication rate, in which dNTPs influence replication rates simply at the level of substrate saturation of polymerase catalytic sites. This model is consistent with observed cell cycle-related variations of dNTP pool sizes. Rate of DNA synthesis is closely coordinated in time with the concentrations of its precursors. Thus, as cycling cells approach S-phase the activities of enzymes of deoxyribonucleotide biosynthesis increase. In particular, the increased activity of ribonucleotide reductase elevates the pool sizes of all four dNTPs.

Might the data from my experiments suggest a particularly strong dependence of replication rate upon a specific deoxyribonucleotide? In my experiments all four dNTP pools declined in parallel with the inhibition of DNA replication at the non-permissive temperature. In addition, the artificial limiting of any dNTP, either by metabolic inhibitors or by exogenous deoxyribonucleosides, is also known to inhibit DNA replication. For example, treatment of cells with

hydroxyurea depletes only purine dNTPs, and this is rapidly followed by inhibition of DNA synthesis (Bianchi *et al.*, 1986a). Moreover, thymidine expands the dTTP pool, which causes allosteric inhibition of the reduction of CDP, thereby inhibiting replication by shrinking the dCTP pool (Bjursell and Reichard, 1973). Therefore, the limited availability of any dNTP seems to influence rate of DNA replication. The dGTP pool is by far the smallest among virtually all mammalian cell lines analyzed to date (Collins and Oates, 1987). Moreover, the dGTP pool was found to fall below the saturating concentration for DNA polymerase α (Leeds *et al.*, 1985; Dresler *et al.*, 1988). The dGTP pool limitation may, then, provide a natural control mechanism in normal DNA replication as a rate-limiting substrate.

Further analysis should focus on rigorously quantitative relation of dNTP pools to the rate of DNA synthesis. Nicander and Reichard (1983) reported that in mouse 3T6 fibroblasts the rate of dTTP pool turnover is equivalent to the rate of DNA replication, showing that the dTTP content of the cell represents a single metabolic pool, all of which is available to the replication apparatus. Our laboratory has confirmed this finding for CHO cells (Leeds and Mathews, 1987). Thus, determination of dTTP specific activity would allow conversion of rates of DNA labeling by thymidine to

time replication rates. The experiment could be designed to shift a mutant culture to the non-permissive temperature, and at intervals to determine replication rate and dNTP pool sizes, to plot the former against the latter values.

Intranuclear dNTP Pools as Determinants of DNA Replication Fidelity

To investigate the control of DNA replication fidelity by its precursors, the biochemical basis of two mutator mutations of CHO cells, Thy^- 49 and Thy^- 303, was analyzed. Both mutant cell lines have imbalanced dNTP pools that result from loss of allosteric regulation of CTP synthetase, and they display elevations in spontaneous mutation frequencies, due to replication errors induced by the dNTP pool biases (Phear *et al.*, 1987; Phear and Meuth, 1989). However, the mutation frequencies differ between the two cell lines by as much as ninefold. When the DNA precursor pools from synchronized, S phase cells were analyzed, to eliminate complexity of interpretation of data by dNTP compartmentation effects, imbalances were observed in extracts of both rapidly isolated nuclei and whole cells. However, while the imbalances in the nuclear extracts were large enough to account for the increase

in mutation frequencies, the biases in the whole-cell extracts were smaller, in both strains. Moreover, the pool ratios in the nuclear extracts were correlated with frequencies of mutations observed in the two mutant cell lines.

As expected, Thy^- 303, which has higher mutation frequencies than Thy^- 49, showed the more aberrant dNTP pools. Thy^- 303 cells contained a higher nuclear dCTP pool size, apparently resulting from differential nuclear compartmentation. Also the dGTP pool was lower in Thy^- 303 cells than in Thy^- 49 cells, possibly due to an additional mutation affecting allosteric control of ribonucleotide reductase during S phase. These pool size changes produced a larger perturbation in the balance of the nuclear dNTP pools in Thy^- 303 cells.

The data suggest (1) that the balance of the four dNTPs influences the DNA replication fidelity to a certain extent, but (2) that the intranuclear balance of DNA precursors is more closely related to the fidelity of DNA synthesis than that in whole-cell extracts, indicating the possibility of nuclear compartmentation of dNTPs.

Conclusion

The results overall support the hypothesis (1) that dNTPs are compartmentalized in the nucleus and (2) that the intranuclear DNA precursors are determinants of the rate and fidelity of DNA replication: while the concentrations of the four dNTPs determine the rate, the balance of the four dNTPs determines the accuracy.

To date, only few laboratories have carried out quantitative studies on dNTP pools in isolated mammalian cell nuclei. Our laboratory devised conditions for extracting nucleotides from nuclei prepared extremely rapidly (Leeds *et al.*, 1985). The dNTP levels in extracts of these rapidly prepared nuclei remain constant over the extraction period, indicating that leakage of intranuclear nucleotides during the isolation procedure is negligible (chapter 3).

When Leeds and Mathews (1987) labeled CHO cells with [^{3}H]cytidine or deoxycytidine, specific activities of dCTP in extracts of both rapidly isolated nuclei and whole cells increased at identical rates, arguing that the intranuclear dNTPs do not constitute a separate metabolic compartment. This finding could also suggest the possibility that dNTPs cross the nuclear membrane by simple diffusion through the nuclear pores. However, my experiments showed that dNTPs are preferen-

tially retained in nuclei under some conditions, suggesting the likelihood that they are tightly bound in the nucleus, prabably to proteins. Equilibration of dCTP pools previously shown might, then, be the result of equilibrium concentrations established by the interactions of proteins that are distributed between the two compartments. Therefore, further studies are needed to define the nature of nuclear compartmentation of dNTPs. Most straightforward could be photoaffinity labeling, using the techniques for identifying nucleotide-binding proteins in subcellular fractions.

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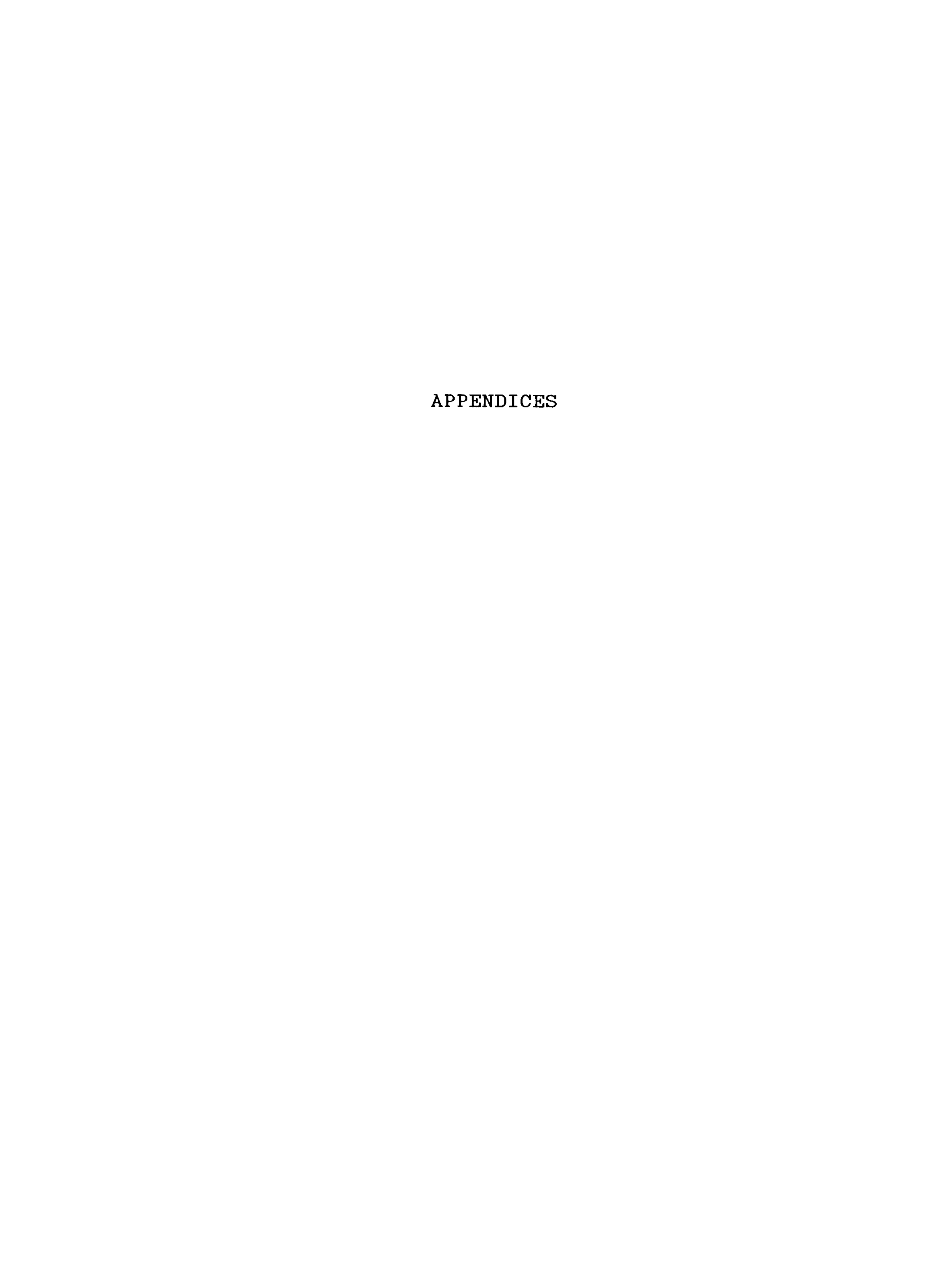

APPENDICES

In the beginning years of my graduate studies, I participated in the projects of Ms. Annelisa Arreco and Dr. Geoffery Sagent, who have worked in our laboratory.

Appendix 1

Deoxyribonucleotide Pools as Targets for Mutagenesis by N-Methyl-N-Nitrosourea

(A-L. Arecco, B-J. Mun, and C.K. Mathews. Mutation Res. 1988. 200:165-175)

Alkylating agents were generally believed to exert their cytotoxic and mutagenic effects at the DNA level (Singer and Grunberger, 1983), i.e., by alkylating particular deoxyribonucleotide residues in DNA. However, Topal and Baker (1982) proposed that these effects could occur at the nucleotide level, i.e., through alkylation of one or more free nucleotides, followed by incorporation of the modified nucleotides into DNA. Working with methyl-labeled N-methyl-N-nitrosourea (MNU), they found that free nucleotides in mammalian cells were far more susceptible to alkylation by this agent than were deoxyribonucleotide residues in DNA. However, they did not ask whether the resultant methylated nucleotides could then be incorporated into DNA

lated nucleotides could then be incorporated into DNA at rates sufficient to account for observed mutation frequencies.

Working with prokaryotic enzymes, others have found that such nucleotides, namely O^6methyl-dGTP and O^4methyl-dTTP, can be incorporated and then stimulate mutagenesis (Eadie et al., 1984; Snow et al., 1984: Preston et al., 1986; 1987; Richardson et al., 1987). These findings suggested that O^6-methylguanine acts primarily by base-pairing with thymidine in double-helical DNA and thereby stimulating GC-to-AT transitions, while O^4-methylthymidine base-pairs with a DNA guanine residue, and hence leads to AT-to-GC transitions.

However, whether significant incorporation of alkylating nucleotides into DNA occurs in mammalian cells was still an open question. Thus, we did a biological test of this question. If a particular nucleotide pool represents a mutagenic target, then transient expansion of that pool should increase the target size and enhance mutagenesis following subsequent treatment with an alkylating agent.

Working either with V79 hamster lung fibroblasts or Chinese hamster embryo fibroblasts (CHEF/18), I attempted to manipulate the target dGTP and dTTP pools with nucleosides and/or metabolic inhibitors. This was

After 8 days of additional culture for recovery of cells and expression of mutations, the 6-thioguanine-resistant mutants in each culture were determined. All genetic work was carried out by Annelisa Arecco. We found that conditions which expand pools of either dTTP or dGTP stimulate mutagenesis by MNU, with the degree of stimulation varying in different experiments from 2- to 6-fold. Thus, our studies suggested that nucleotide pools represent significant biological targets for alkylating agents.

Appendix 2

Ribonucleotide Reductase: A Determinant of 5-Bromodeoxy-Uridine Mutagenesis in Phage T4
(R.G. Sargent, J. Ji, B. Mun, C.K. Mathews.
Mol. Gen. Genet. 1989. 217:13-19).

Mutagenesis by 5-bromodeoxyuridine (BUdR) can result from base-pairing errors either during replication of a BUdR-containing template or at the nucleotide incorporation step. Replication errors give rise predominantly to AT-to-GC transitions, while incorporation errors, in which BrdUTP, competes with dCTP at a template guanine site, should give rise to GC-to-AT transitions.

However, since BUdR mutagenesis was found to be highly concentration-dependent (Kaufman and Davidson, 1978; Ashman and Davidson, 1981), Hopkins and Goodman (1980) proposed that BUdR mutagenized largely by incorporation errors. They suggested that BrdUTP, acting like dTTP, could inhibit the CDP reductase activity of ribonucleotide reductase, and hence, shrink the pool of dCTP. BrdUTP could then compete effectively with the depleted dCTP, for incorporation opposite template dGMP. If so, this pathway should be sensitive to deoxyribonucleoside triphosphate (dNTP) pool fluctuations. Since dNTP pools are regulated through allosteric control of ribonucleotide reductase, the control of this enzyme should be a determinant of BUdR mutagenesis (Hopkins and Goodman, 1980)--if mutagenesis results largely from incorporation errors.

To test this proposal, we established conditions under which T4 phage DNA replication is dependent upon feedback-sensitive ribonucleotide reductase of *E. coli*, since T4 phage-encoded reductase is insensitive to feedback inhibition (Berglund, 1972). Geoff Sargent and Jiuping Ji examined BUdR mutagenesis of rII mutants known to revert to wild type either by AT-to-GC or GC-to-AT transition pathway. When the phage reductase was functioning, BUdR preferentially stimulated GC-to-AT transition mutagenesis, while substitution of the host

reductase specifically stimulated AT-to-GC transitions. These results confirm that ribonucleotide reductase is a determinant of BUdR mutagenesis. However, in my experiments BUdR had relatively small effects upon dNTP pool sizes. This suggested that the relationship between deoxyribonucleotide metabolism and BUdR mutagenesis is more complex than anticipated, while the data do not rule out effects upon dNTP levels in the microenvironment of the replication fork (Mathews and Sinha, 1982).