AN ABSTRACT OF THE THESIS OF

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Christopher K. Mathews

Deoxyribonucleoside triphosphate (dNTP) concentrations measured in cells are not symmetric. dGTP almost always represents only 5-10% of the total dNTP pools in cells. In an *in vitro* replication system involving semiconservative replication from an SV 40 origin, the mutation frequency of an M13 phagemid replicated by human cell extracts in reaction mixtures containing "biologically biased" dNTP pools estimated from HeLa cell nuclei is not significantly different from that seen when replication is done with equimolar dNTP concentrations. Significant reduction of dGTP pool while keeping other dNTPs at "biologically biased" dNTP concentrations during replication reaction also did not increase mutation frequency. In contrast, *in vitro* replication with dNTP concentrations calculated from normal diploid fibroblast cells, which are three- to four-fold lower in concentrations, showed a marked reduction of the observed mutation frequency, showing the importance of overall dNTP levels during replication on mutation frequency *in vitro*. When whole-cell dNTP concentrations in HeLa cells were measured during S-phase, dNTP levels underwent a transient decrease in the middle of S-phase. Average HeLa cells' dNTP levels were also found to correlate with average DNA accumulation rates during S-

phase, although no detailed relationship can yet be deducted from the available data. No significant changes in the ratio of the four dNTP concentrations were found during S-phase. Mutation rates of green fluorescent protein (GFP) inserted in either middle or late-replicating region of a chromosome in HeLa cells also correspond to average DNA accumulation rates and dNTP levels during middle and late S-phase. The late-replicating GFP-HeLa cells have a higher mutation rate than the middle-replicating GFP-HeLa cells, as the average DNA accumulation rates and dNTP pool levels were also lower in middle compared to late S-phase. Taken together, these observations indicate that dNTP levels could play a role in determining the S-phase DNA replication rate and also the replication fidelity in mammalian cells.

Modulation of Deoxyribonucleoside Triphosphate Levels, DNA Synthesis Rates and Fidelity in Mammalian Cells

by
Stella A. Martomo

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Stella A. Martomo, Author

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For the loving memory of my father,
Augustinus Martomo
1943-2000

MODULATION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATE LEVELS, DNA SYNTHESIS RATES AND FIDELITY IN MAMMALIAN CELLS

CHAPTER 1

INTRODUCTION

The careful balance between genomic stability and genetic variation in living organisms is the basis of life's existence. Continued survival of organisms requires faithful duplication of the genome. On the other hand, mutations, although some may be detrimental, provide a source for genetic variations which are important in evolutionary adaptation.

The spontaneous mutation rate in mammalian and most microbial cells is estimated to be less than one mutation per billion base pairs per cell generation (Kunkel, 1992a). Contributing to this low mutation rate is the high fidelity of DNA replication process and the variety of DNA repair mechanisms. DNA replication multi-protein complexes, as first elements responsible for the low spontaneous mutation rate, rapidly duplicate large amounts of genetic material with very high accuracy. The DNA polymerase substitution error rate can be as low as one in ten million base pairs incorporated (reviewed in Kunkel, 1992b). Several factors contribute to the high fidelity of DNA polymerases. Replicative DNA polymerases insert only about one incorrect nucleotide opposite a template for every tens of thousands of correctly inserted bases. The exonucleolytic proofreading activity associated with some DNA polymerases can cleave the incorrectly base-paired template and inserted nucleotide during replication. In general, the proofreading activity contributes about a hundred-fold further error reduction to achieve the low substitution error rate (reviewed in Echols and Goodman, 1991).

DNA repair proteins contribute significantly to the low spontaneous mutation rate. DNA mismatch repair proteins correct mismatches that are present in newly replicated DNA (reviewed in Modrich, 1991). Another repair protein, the uracil-DNA glycosylase, which excises uracil from DNA, was reported to be present in the replication foci (Otterlei *et al.*, 1999), suggesting its role in repair of newly replicated DNA. Still other repair proteins are available to correct the variety of premutagenic lesions present in DNA to ensure accurate genome duplication.

As reviewed in Kunz and Kohalmi (1991), deoxyribonucleoside triphosphates (dNTPs) as building blocks for DNA synthesis play a fundamental role in DNA replication fidelity. The availability of balanced supplies of dNTPs during replication has been shown to affect the DNA polymerase fidelity *in vitro* (reviewed in Kunkel, 1992a). Perturbation of "normal" dNTP pool concentrations *in vivo* has been documented to produce a range of genetic effects associated with inaccurate DNA repair and replication. However, dNTP pool measurements done in the Mathews laboratory and elsewhere revealed that "normal" dNTP pools are asymmetric in a variety of cells that have been studied (Mathews and Ji, 1992). In prokaryotes, the dNTP pool measured or "bulk" dNTP pools is not thought to be the "replication-active" pool. The measured dNTP concentrations do not reflect the effective dNTP concentrations seen by the DNA polymerase replication machinery. In eukaryotic cells, it is still not completely understood whether there are "replication-active" pools that are different from the measured dNTP pools (reviewed in Mathews and Ji, 1992). In mammalian cells, which are the focus of this thesis, deoxyguanosine triphosphate (dGTP) is almost always underrepresented among all other dNTP pools measured. The existence of the asymmetric pools poses questions regarding the possible contribution of the pools on the spontaneous mutation rate. The relationship between dNTP levels and DNA replication fidelity *in vitro* and *in vivo* in mammalian cells is the main focus of this thesis.

The following is a summary of processes involved in mammalian chromosomal DNA replication as currently understood. Some aspects, such as the

kinetic mechanism of DNA replication fidelity, have been taken from studies involving prokaryotic DNA polymerases since the details of the kinetic mechanism in mammalian DNA polymerases have not been elucidated.

BACKGROUND

DEOXYRIBONUCLEOSIDE TRIPHOSPHATES

dNTP pool measurement serves as quantitation of dNTP concentrations in cells at the time of extraction. Each dNTP concentration measured is determined by the rate of its production less the rate of its utilization in DNA replication and turnover. DNA replication is almost exclusively the only utilization pathway for dNTPs. However, at high concentration dATP has also been shown to participate in the caspase-3 activation to induce apoptosis (Leoni *et al.*, 1998, Oliver *et al.*, 1996). dNTP production in cells is achieved through two pathways: *de novo* synthesis and salvage pathway.

De Novo Synthesis of Deoxyribonucleotides

Synthesis of nucleotides from low-molecular-weight precursors such as CO_2, NH_3, ribose phosphate and certain amino acids is termed *de novo* pathways (Kornberg and Baker, 1992, Mathews *et al.*, 2000). Figure 1.1 illustrates the overview of nucleotide metabolism taken from Mathews (1997).

The pathways for *de novo* nucleotide synthesis are generally conserved in all organisms studied. As there are two major classes of nucleotides, purines and pyrimidines, *de novo* synthesis is also divided into two major categories, the purine

nucleotide metabolism which produces adenine and guanine nucleotides, and the pyrimidine nucleotide metabolism which produces cytidine and thymine nucleotides needed for DNA replication.

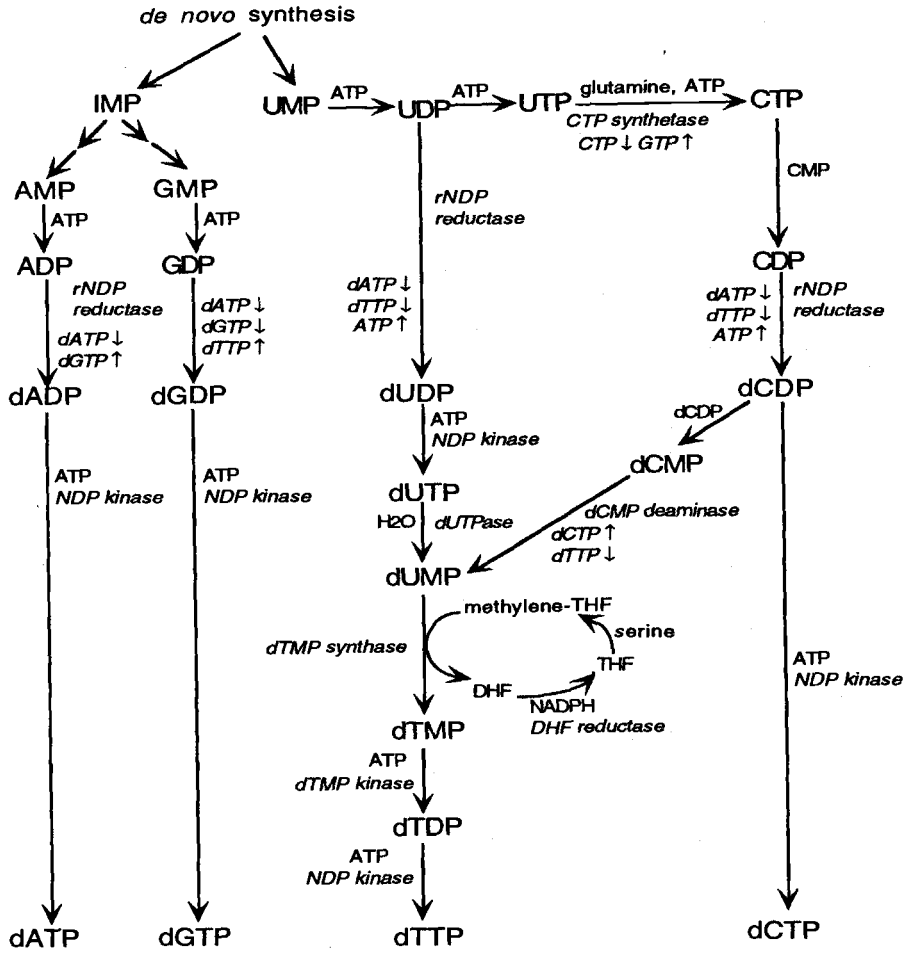

Figure 1.1. Overview of Nucleotide Synthesis. This schematic diagram is taken from Mathews (1997). Upward and downward arrows represent allosteric activators and inhibitors respectively.

The enzyme 5-phospho-α-D-ribosyl-1-pyrophosphate (PRPP) amidotransferase, which is feedback regulated by inosine 5'-monophosphate (IMP), adenosine 5'-monophosphate (AMP), guanosine 5'-monophosphate (GMP) and its

substrate PRPP, is the initial commitment to purine biosynthesis. PRPP synthase, the enzyme controlling intracellular contents of PRPP, has also been reported to be regulated by the end products of *de novo* purine biosynthesis (Becker and Kim, 1987). Human PRPP synthase exists in at least two different isoforms (Roessler *et al.*, 1990). An associated PRPP synthase-associated protein of 39 kDa (PAP39) affects the catalytic activity of the enzyme, and both PRPP synthase and PAP39 is expressed differentially in different tissues, indicating different regulation of enzyme in different tissues (Tatibana *et al.*, 1995).

A trifunctional enzyme involved in the next three steps of *de novo* purine biosynthesis has been isolated from human cells (Poch *et al.*, 1998). This trifunctional enzyme encodes glycinamide ribonucleotide synthase, aminoimidazole ribonucleotide synthase and glycinamide ribonucleotide transformylase (GAR Tfase), and there is evidence that the expression of this multifunctional protein is differentially regulated during tissue development (Brodsky *et al.*, 1997). Another multi-functional enzyme in purine *de novo* biosynthesis isolated from human cells is 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase (AICAR Tfase/IPCHase) (Rayl *et al.*, 1996, Yamauchi *et al.*, 1995, Szabados *et al.*, 1994). In addition to the multi-functional enzymes, isolation of a large complex of purine biosynthetic enzymes involving folate enzymes GAR Tfase, AICAR Tfase, and also including serine transhydroxymethylase and enzymes for synthesis of 10-formyl tetrahydrofolate and other folate cofactors from chicken liver has also been reported (Caperelli *et al.*, 1980).

The second point of regulation of purine biosynthesis is at the brach point of either ATP or GTP formation from IMP. Adenylate synthesis by adenylosuccinate synthase requires GTP as cofactor, and it is up-regulated by GTP (Van der Weyden and Kelly, 1974). On the other hand, the rate of inosinate conversion to GTP is regulated by ATP level (Holmes *et al.*, 1974).

The human genes encoding the first three enzymes involved in pyrimidine synthesis, carbamoyl phosphate synthase, aspartate carbamoyl transferase, and

dihydroorotase have been cloned (Iwahana *et al.*, 1996), and they are linked together to form one complex polypeptide (termed CAD). The trifunctional enzyme is regulated both at the transcriptional and post-transcriptional levels (Rao and Church, 1988), and it has been shown that the first enzyme in the complex polypeptide, carbamoyl phosphate synthase, is allosterically regulated (Tatibana and Shigesada, 1972, reviewed in Jones, 1980) as well as activated by cAMP-dependent protein kinase A (PKA) phosphorylation (Carrey *et al.*, 1985). CAD is also regulated by mitogen-activated protein (MAP) kinase cascade (Graves *et al.*, 2000). CAD level has been shown to be correlated with cell growth, and its level is highest at the G1-phase of the cell cycle (Rao and Church, 1988, Morford *et al.*, 1994).

The second multifunctional enzyme involved in pyrimidine biosynthesis is UMP synthase, which is composed of orotate phosphoribosyl transferase and orotidylate decarboxylase. The human UMP synthase bifunctional protein has been cloned (Suttle *et al.*, 1988). Although it is thought that the multifunctional enzymes channelled the five intermediates of pyrimidine biosynthesis, carbamoyl phosphate, carbamoyl aspartate, dihydroorotate, orotate, and orotidylate, there is evidence to suggest that channelling is not efficient in the UMP synthase bifunctional enzyme (Traut, 1989). Each of the catalytic sites in the human UMP synthase bifunctional enzyme is more stable when covalently linked (Yablonski *et al.*, 1996), suggesting a structural role for the existence of the multienzyme complex.

The next step for UMP, AMP and GMP is the reversible conversion of nucleoside monophosphate to diphosphate, and human tissues contain a uridylate-cytidylate kinase (UMP-CMPK), five isozymes of adenylate kinase (AK) and several guanylate kinase (GUKs) (reviewed in Van Rompay *et al.*, 2000). These kinases are mostly specific for each base but not very specific to the sugar. Ribonucleoside diphosphates are then reduced to deoxyribonucleoside diphosphates by ribonucleotide reductase, a key enzyme in nucleotide metabolism, and finally deoxyribonucleoside diphosphates are converted to triphosphates by nucleoside diphosphate kinase, which catalyses reversible phosphate exchanges between

nucleoside diphosphates and triphosphates (reviewed in Lascu, 2000). Six human isoforms of nucleoside diphosphate kinase (NM23-H1 to NM23-H8) exist (Lacombe *et al.*, 2000), and NM23-H1 and NM23-H2 have been implicated in metastasis (Stahl *et al.*, 1991) and pathogenesis (Hailat *et al.*, 1991, Zhang *et al.*, 1997) of tumors (reviewed in Veron *et al.*, 1994).

Ribonucleotide reductase (RNR) is the rate-limiting enzyme in nucleotide biosynthesis for DNA replication (Reichard, 1993), and it is allostericaly regulated so that its activity corresponds to the needs of dNTPs for DNA synthesis (Hendricks and Mathews, 1998, Chimploy and Mathews, 2001). Mammalian RNR is composed of two nonidentical subunits: R1, which contains redox-active disulfides active site and two different allosteric nucleoside triphosphate binding sites (Thelander and Reichard, 1979, reviewed in Jordan and Reichard, 1998), and R2, which has the essential iron center-generated tyrosyl free radical (Thelander *et al.*, 1985). Binding of ATP to one of the allosteric sites, the activity site, activates the enzyme, while binding of dATP to the same site inhibits all four activities. Binding of ATP, dATP, dTTP or dGTP to the other allosteric site, the substrate specificity site, governs the activity of the enzyme for specific substrates (Thelander and Reichard, 1979). ATP stimulates the reduction of UDP and CDP, dTTP stimulates reduction of GDP, and dGTP stimulates ADP reduction. RNR activity in cells is regulated throughout the cell cycle (Engstrom *et al.*, 1985). The enzyme activity increases as the cell proceeds through S-phase. Transcription of both R1 and R2 is specific to S-phase (Bjorklund, 1990). However, the level of R1 protein, which has a half-life of more than 20 hours, has been shown to be constant throughout the cell cycle (Engstrom *et al.*, 1985, Mann *et al.*, 1988). R2 protein, with a short half-life of 3 hours, has been shown to be specifically stabilized in S-phase and degraded in late mitosis (Chabes and Thelander, 2000), and since R2 is limiting for activity, it confers the cell cycle regulated activity of RNR (Eriksson *et al.*, 1984). Although increased RNR activity is observed in S-phase, it has been shown that overproduction of R2 during S-phase does not result in an increase in dNTP pools (Akerblom *et al.*, 1981), stressing the

importance of allosteric control of RNR (Weinberg *et al.*, 1981) together with dNTP degradation pathways in keeping a tight control on the level of dNTP pools in cells (reviewed in Reichard, 1988). R1 and R2 are undetectable in quiescent or terminally differentiated cells (Mann *et al.*, 1988). However, p53-inducible R2 has been shown to be able to function with R1, and UV irradiation of G_0/G_1 mouse fibroblast cells results in expression of R1 protein, indicating the role of R1/p53R2 in damage-induced nucleotide synthesis in resting cells (Guittet *et al.*, 2001).

Synthesis of thymidylate is achieved through either the action of dCMP deaminase or dUTPase resulting in dUMP (Fig. 1.1.). dCMP deaminase (Ellims *et al.*, 1981, Ellims *et al.*, 1983, Weiner *et al.*, 1993, Weiner *et al.*, 1995) supplies most of the dUMP in mammalian cells. This enzyme is allostericaly controlled; it is activated by dCTP and inhibited by dTTP (Ellims *et al.*, 1983). dUMP is then converted to thymidylate by the enzyme thymidylate synthase (TS), which acts by transferring a methylene group from methylene tetrahydrofolate (THF) to deoxyuridylate accompanied by the reduction of the group to CH_3 (reviewed in Carreras and Santi, 1995, Montfort and Weischsel, 1997). Regeneration of THF is by dihydrofolate reductase and this regeneration process is essential in sustaining thymidylate synthesis. Translation of human TS mRNA is regulated by its own protein product through binding of TS protein to at least two distinct sequences on its own mRNA that results in translational repression (Chu *et al.*, 1991, Chu *et al.*, 1993). In addition, there is evidence to suggest that TS protein regulates the expression of p53 through the same translational repression, overexpression of TS protein decreases the translational efficiency of p53 mRNA, suggesting a role for TS in cellular gene expression (Ju *et al.*, 1999).

Salvage Pathway for Synthesis of Deoxyribonucleotides

Salvage pathways utilize broken down nucleotides from degraded DNA or RNA in the form of nucleobases to form nucleotides. The salvage pathway is physiologically important in resting cells, such as most mammalian tissues, since *de novo* synthesis of nucleotides is lacking in these cells (reviewed in Arner and Eriksson, 1995). The importance of the salvage pathway in cycling cells has also been implicated. Cohen *et al.* (1983) have shown that in S-phase thymocytes *de novo* synthesis is responsible for production of purine nucleotides, whereas salvage pathway predominantly supplies the pyrimidine nucleotides for DNA synthesis. The enzymes involved in catabolism of nucleotides and salvage pathway are also important in regulating the levels of intracellular dNTP pools (Reichard, 1988).

There are several pathways for salvage of nucleotides. The first is direct conversion of a nucleoside to a nucleotide through kinases. In human cells deoxycytidine kinase (Bohman and Eriksson, 1988, Datta *et al.*, 1989a,b), thymidine kinase 1 (Sherley and Kelly, 1988, Munch-Petersen *et al.*, 1991), thymidine kinase 2 (Eriksson *et al.*, 1991, Munch-Peterson *et al.*, 1991, Jansson *et al.*, 1992), deoxyguanosine kinase (Yamada *et al.*, 1982, Sarup and Fridland, 1987) and adenosine kinase (Yamada *et al.*, 1981) are responsible for conversion of nucleosides to nucleotides. Deoxycytidine kinase (dCK) phosphorylates dCyd, dAdo and dGuo (Datta *et al.*, 1989b, Kim and Ives, 1989). While the cell cycle variation in dCK activity is different in different cell lines, dCK mRNA level was mostly constant, and therefore, dCK is thought to be constitutively expressed during the cell cycle in most cell lines studied (Hengstschlager *et al.*, 1993). Thymidine kinase 1(TK1) phosphorylates Thd and dUrd (Eriksson *et al.*, 1991, Munch-Petersen *et al.*, 1991). TK1 activity is closely correlated with S-phase (Brent, 1971, Pegoraro and Bernengo, 1971, Taylor *et al.*, 1972, Bello, 1974) with evidence that transcription factor E2F regulates the S-phase dependent expression of TK1 (Ogris *et al.*, 1993). In addition, tight regulation mechanisms of TK1 throughout cell cycle by

transcriptional, translational and post-translational regulatory mechanism have been demonstrated (Stewart *et al.*, 1987, Kauffman and Kelly, 1991, Chang and Huang, 1993, Chang *et al.*, 1994). Thymidine kinase 2 (TK2) is a mitochondrial enzyme encoded by a nuclear gene (Attardi and Schatz, 1988, Hartl and Neupert, 1990). It phosphorylates Thd, dCyd, and dUrd (Eriksson *et al.*, 1991, Munch-Petersen *et al.*, 1991, Jansson *et al.*, 1992). This enzyme is not cell-cycle-regulated (Kit, 1985). Deoxyganosine kinase (dGK) is a mitochondrial enzyme (Gower *et al.*, 1979), and it phosphorylates dGuo and dAdo (Yamada *et al.*, 1982, Sarup and Fridland, 1987, Wang *et al.*, 1993). Human dGK has been shown to be homologous in sequence to human dCK (Johansson and Karlsson, 1996).

The conversion of nucleoside monophosphate to nucleoside through the action of 5'-nucleotidases, plays an important role in fine tuning the intracellular deoxyribonucleotide pool levels (Chan *et al.*, 1974, Reichard, 1988, Hoglund and Reichard, 1990a,b). Three important classes of the 5'-nucleotidase in higher organisms include the cytosolic high K_M nucleotidase (hkm-NT) (Itoh, 1981), the cytosolic 5'(3')-deoxynucleotidase (dNT-1) (Fritzon and Smith, 1971) and the mitochondrial enzyme (dNT-2) (Henke *et al.*, 1989, Rampazzo *et al.*, 2000). Recently it was shown that 5'(3')-deoxynucleotidase (dNT-1), which preferably dephosphorylates pyrimidine deoxyribonucleotides 5'-monophosphates (Hoglund and Reichard, 1990a), is involved in the substrate cycles regulating pyrimidine nucleotide pools in human 293 and hamster V79 cells (Gazziola *et al.*, 2001). The high K_M 5'-NT may function as phosphotransferase (Worku and Newby, 1982) supplementing the functions of the deoxyribonucleoside kinases in the salvage pathway (Arner and Eriksson, 1995).

Also important is the reversible conversion of base to nucleosides through phosphorylases. In humans, the enzymes in this class include purine nucleoside phosphorylase (Stoeckler *et al.*, 1978, Zannis *et al.*, 1978), uridine phosphorylase (Watanabe and Uchida, 1995, Liu *et al.*, 1998) and thymidine phosphorylase (Kubilus *et al.*, 1978, Desgranges *et al.*, 1981). Although the regulatory mechanisms

of these enzymes are not as clear, the metabolic roles of the enzymes have been studied extensively. Deficiency in purine nucleoside phosphorylase leads to impairment of T-cell function (reviewed in Bzowska *et al.*, 2000). It was shown that purine nucleoside phosphorylase synthesis is upregulated significantly in the early stages of T-cell transformation (Neote *et al.*, 1985). Mutations in the thymidine phosphorylase gene has been shown to be responsible for mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) (Nishino *et al.*, 1999, Nishino *et al.*, 2001). Increase in thymidine pool is thought to result in mitochondrial nucleotide pools imbalance leading to the mitochondrial DNA alterations observed in MNGIE.

Other important salvage pathways include interconversion by base alteration by deaminases, and direct conversion of a base into a ribonucleotide through phosphoribosyl transferases. An example of deaminases is human adenosine deaminase, which presents in multiple molecular forms in human tissue (Van der Weyden and Kelley, 1976). Defects in this enzyme have been implicated in severe immunodeficiency (reviewed in Hirschhorn, 1990). An example of the transferases is hypoxanthine-guanine phosphoribosyl transferase (HGPRTase). Deficiency in human HGPRTase has been implicated in Lesch-Nyhan syndrome (LNS), characterized by excessive purine production and severe neurological manifestation (Wilson and Kelley, 1983, reviewed in Stout and Caskey, 1985).

DNA REPLICATION

The process of chromosomal DNA replication in mammalian cells is compartmentalized within the nucleus. Therefore, DNA replication is partitioned from the cytoplasm, which is the site of synthesis of proteins and other cofactors that regulate and function in DNA synthesis (reviewed in Malkas, 1998). Another aspect that adds to the complexity of mammalian DNA replication is that the mammalian chromosome is a complex nucleoprotein structure composed of both DNA and

protein (reviewed in Huberman, 1973). Mammalian chromosomes also contain multiple replication origins per DNA molecule (Huberman and Riggs, 1966). It can be envisioned that the DNA replication process in mammalian cells is a highly coordinated and complex process; however, it is not thoroughly understood.

Proteins Involved in Mammalian DNA Replication

DNA Polymerases

In eukaryotes, DNA polymerases α with the tightly associated primase activity, δ and ε are the major replicative DNA polymerases (reviewed in Stillman, 1994). However, pol ε was found to be present only during *in vivo* replication and not in the SV 40 *in vitro* replication system used to study mammalian DNA replication. All of the replicative DNA polymerases have one large subunit that contains the polymerase active site and, with the exception of pol α-primase, the same subunit or an associated polypeptide carries a proofreading 3'→5' exonuclease (reviewed in Baker and Bell, 1998). This 3'→5' exonuclease activity contributes about three orders of magnitude to the fidelity of DNA replication (Kunkel, 1988). Sequence alignments, structural studies, and site-directed mutagenesis studies indicate that the exonuclease and the polymerase active sites of these enzymes can be considered largely independent catalytic modules (Joyce and Steitz, 1994). The mechanism of exonucleolytic proofreading is best understood for *E. coli* DNA polymerase I (pol I) (Freemont *et al.*, 1988, Joyce and Steitz, 1994). In *E. coli* DNA pol I, the polymerase and exonuclease active site are separated by 30 A^{o} but linked by a shared binding cleft. A properly base-paired template would be preferred for polymerization, while an unmatched terminus would result in fraying and switching of the single-strand to the exonucleolytic site.

The list of mammalian enzymes known to catalyze polymerisation of DNA has grown remarkably longer in the last couple of years. Although mammalian chromosomal replicative DNA polymerases are still believed to be DNA polymerases α, δ and ε, new subsets of DNA polymerases are also thought to participate in S-phase replication in the event of DNA damage or misalignment of primer-template junction, therefore, ensuring the completion of S-phase. Some of the polymerases are capable of traversing past lesions accurately while others with less accuracy. It is still a question of how all of the different DNA polymerases can access each of its specialized substrate, such as specific DNA lesions, during DNA synthesis (reviewed in Sutton and Walker, 2001). Adding to the complexity, some of the newly discovered polymerases do not yet have clearly understood functions.

Mammalian DNA Polymerases α, δ and ε

DNA pol α is one of the major polymerases involved in mammalian DNA synthesis (reviewed in Wang, 1991). The structure of the whole complex and of the individual subunits in this DNA polymerase-primase complex seems to be conserved in all eukaryotes studied. Human pol α consists of four subunits: the subunit associated with the polymerase activity, which is about 165 kDa (Wang *et al.*, 1989), a 70-kDa essential subunit of unknown function and the primase subunits of 58 kDa and 48 kDa (Wang *et al.*, 1984). The two small subunits, which constitute the primase activity, are responsible for synthesizing small RNA primers of about 10 nucleotides in length. It is because of the tight association of the polymerization and priming functions that pol α is more correctly referred to as DNA polymerase-primase. The mouse primase gene is induced during transition from quiescence to proliferation, and in actively proliferating cells the gene is constitutively expressed throughout the cell cycle (Tseng *et al.*, 1989). Human DNA polymerase α is phosphorylated in a cell-cycle-dependent manner (Nasheuer *et al.*, 1991); the

catalytic polypeptide is found to be phosphorylated throughout the cell cycle and hypophosphorylated in mitotic phase, whereas the 70-kDa subunit is phosphorylated only in mitotic phase. Cell-cycle dependent phosphorylation of the 58- and 48-kDa subunits were not observed. The cyclin-dependent phosphorylation of DNA pol α is suggested to affect the regulation of the initiation of DNA replication (Schub *et al.*, 2001).

Human DNA pol δ is a heterodimeric protein complex composed of two subunits of approximate molecular weight 125 and 50 kDa (Lee *et al.*, 1991). The larger 125-kDa subunit is associated with both the DNA polymerase activity and the proofreading 3'→5' exonuclease activity (Lee *et al.*, 1991, Simon *et al.*, 1991). Proliferating cell nuclear antigen (PCNA) is reported to interact with the N-terminal region of the 125-kDa subunit (Zhang *et al.*, 1995). Pol δ is a major replicative polymerase and is required for DNA synthesis of both the leading and lagging strands during SV 40 replication *in vitro* as confirmed in a UV-crosslinking study (Zlotkin *et al.*, 1996). Common characteristics that are used to identify mammalian pol δ are the proofreading 3'→5' exonuclease activity (Kunkel *et al.*, 1987), sensitivity of enzyme to the drug aphidicolin, low polymerase processivity in the absence of PCNA (Tan *et al.*, 1986, Syvaoja *et al.*, 1990), and resistance to the pol α-specific drug N2-(*p-n*-butylphenyl)-2'-deoxyguanosine 5' triphosphate (BuPhdGTP) (Lee *et al.*, 1985) (reviewed in Burgers, 1989).

The human form of pol ε consists of two subunits of approximate molecular weight 250 and 55 kDa (Syvaoja and Linn, 1989, Lee and Toomey, 1987, Syvaoja *et al.*, 1990). Its involvement in chromosomal DNA replication is suggested by a UV-crosslinking study that found that pol ε existed in replicating chromosomal DNA, although pol ε does not seem to be required for SV 40 DNA replication (Zlotkin *et al.*, 1996). Pol ε does have proofreading exonuclease activity and is distinguished from pol δ because of its lack of stimulation by PCNA (Syvaoja *et al.*, 1990, Lee *et*

al., 1991). The level of mRNA expression of pol ε in cells is strongly correlated with cell proliferation (Kesti *et al.*, 1993).

Mammalian DNA Polymerase ζ

DNA polymerase ζ (pol ζ) is one of the newly discovered polymerases, and it is in the same class-B family of DNA polymerases as pol α, δ and ε (reviewed in Burgers *et al.*, 2001). Pol ζ is an error-prone DNA polymerase that is induced during DNA damage, and recently it has been implicated to act together with pol ι (see *UmuC-DinB-Rad30-Rev1 Superfamily of DNA Polymerases*) to bypass DNA lesions (Johnson *et al.*, 2000).

Mammalian DNA Polymerase β

Human DNA polymerase β (pol β) is a polypeptide with molecular weight of 39 kDa (Mullen and Wilson, 1997). Pol β does not have a proofreading exonuclease activity, and kinetic analysis has shown it to be a distributive enzyme. However, short gaps with 5'-phosphate termini are filled processively (Singhal and Wilson, 1993). This enzyme is thought to function in short-patch repair, including base-excision repair.

Mammalian DNA Polymerase λ and μ

Mammalian DNA polymerase λ (pol λ) and μ (pol μ) belong in the X-class of DNA polymerases together with pol β (reviewed in Burgers *et al.*, 2001). Human pol λ has

been shown to have 5'-deoxyribose-5-phosphate lyase (dRP lyase) activity, suggesting its role in single-nucleotide base excision repair in mammalian cells (Garcia-Diaz *et al.*, 2001). Pol μ has been suggested to participate in somatic hyper-mutation of immunoglobulin genes (Dominguez *et al.*, 2000, reviewed in Ruiz *et al.*, 2001).

Mammalian DNA Polymerase σ

Human has two genes for DNA polymerase σ (pol σ). Pol σ is distantly related to pol X superfamily of DNA polymerases, and it (TRF4) is required for sister chromatid cohesion (Burgers *et al.*, 2001, Sutton and Walker, 2001).

Mammalian DNA Polymerase γ

Human DNA polymerase γ (pol γ) was purified from HeLa cells, and it consists of two subunits of molecular weight 140 and 54 kDa (Gray and Wong, 1992). The catalytic polymerase activity resides in the larger 140-kDa subunit. Pol γ is located in mitochondria, and it is the mitochondrial replicative DNA polymerase (Bolden *et al.*, 1977, reviewed in Wang, 1991). This enzyme has a proofreading 3'→5' exonuclease activity (Gray and Wong, 1991, Kunkel and Soni, 1988, Kunkel and Mosbaugh, 1989).

UmuC-DinB-Rad30-Rev1 Superfamily of DNA Polymerases

The newly described UmuC-DinB-Rad30-Rev1 superfamily of DNA polymerases have been identified to exist in both prokaryotes and eukaryotes. Most

of these polymerases are known to function in translesion synthesis to allow damaged templates during replication to be bypassed and hence ensuring S-phase progression (reviewed in Sutton and Walker, 2001). Human pol η, encoded by hRAD30A/XP-V gene, and pol ι, encoded by the hRAD30B gene, are members of the RAD30 subfamily. Biochemical characteristic of human pol η indicates that it is able to bypass cis-syn cyclobutane pyrimidine dimers accurately by inserting two adenines opposite the lesion (Nelson *et al.*, 1996a, Johnson *et al.*, 2001). Pol ι has been shown to have a 5'-deoxyribose phosphate lyase activity implicating its role in base excision repair (Bebenek *et al.*, 2001). Pol κ, encoded by the hDINB1 gene, is another polymerase in this superfamily. Rev1p or deoxycytidyl transferase is a related enzyme to the superfamily, and it functions in translesion synthesis (Nelson *et al.*, 1996b). Pol κ is suggested to have a role in spontaneous mutagenesis due to its low fidelity but moderate processivity (Ohashi *et al.*, 2000).

Other Mammalian DNA Polymerases

Other mammalian DNA polymerases that are newly discovered include pol θ which possibly functions in repair of DNA cross-links (reviewed in Sutton and Walker, 2001).

Other Proteins in Mammalian Replication Forks

The replication fork contains several key proteins other than DNA polymerases that synthesize new strands and the editing exonuclease associated with the replicative polymerase. These proteins include the accessory proteins that control interaction of the polymerases with the DNA and the helicase that melts the double

helix to generate a replication fork (Baker and Bell, 1998). Enzymes present in the eukaryotic replication fork have been identified through *in vitro* replication studies using mammalian cell extracts that support complete replication of plasmid DNA containing the SV 40 replication origin (Li and Kelly, 1984). This *in vitro* replication reaction requires only one viral protein, SV 40 Large T Antigen (Li and Kelly, 1984). This system has led to identification of several proteins required in mammalian DNA replication (reviewed in Waga and Stillman, 1998, Hickey and Malkas, 1997).

Replication Protein A

Human replication protein A (RPA) is a single-stranded DNA-binding protein that exists as a heterotrimeric complex consisting of subunits with apparent masses of approximately 70, 34, and 11 kDa (Wold and Kelly, 1988, Fairman and Stillman, 1988, reviewed in Waga and Stillman, 1998). p70 binds to the primase subunits of pol α-primase and the heterotrimeric complex binds to SV 40 T Antigen, both in support of the interaction required for the assembly of the primosome (Kenny *et al.*, 1990). The human p70 alone can bind single-stranded DNA, but it cannot support DNA replication *in vitro*. The 32-kDa subunit has been observed to show an S-phase-specific phosphorylation suggesting cell-cycle dependent regulation of RPA (Din *et al.*, 1990).

Replication Factor C

Replication factor C (RFC) is conserved in all eukaryotes studied. It is one of the key proteins involved in loading the replicative polymerases to create the replication fork (reviewed in Waga and Stillman, 1998). Human RFC is a complex of

five subunits, p140, p40, p38, p37, and p36 (Bunz *et al.*, 1993, Pan *et al.*, 1993), and it preferentially binds to primer-template junction created by the annealing of an oligonucleotide to single-stranded DNA, or by synthesis of a DNA primer on a single stranded DNA template or nicked duplex DNA (Tsurimoto *et al.*, 1990). It acts as DNA-dependent ATPase that is stimulated further by PCNA (Tsurimoto *et al.*, 1990). RFC's main role in replication is to load the trimeric, ring-like structure of PCNA onto DNA at a primer-template junction or to load it onto a nicked site in duplex. RFC-catalyzed PCNA loading is a necessary event preceding the assembly of pol δ onto template DNA to form a processive holoenzyme (Tsurimoto and Stillman, 1991, Waga and Stillman, 1994). Mutational analysis shows distinct regions of the p140 subunit that are responsible for DNA and PCNA binding (Fotedar *et al.*, 1996, Mossi *et al.*, 1997). The subunits of RFC show very high sequence and functional similarity to replication proteins known in *E. coli* and bacteriophage T4 as clamp-loading proteins (O'Donnell *et al.*, 1993, reviewed in Waga and Stillman, 1998).

Proliferating Cell Nuclear Antigen

Proliferating cell nuclear antigen (PCNA) is the DNA polymerase clamp. It forms a homotrimeric complex and functions as a DNA polymerase accessory factor (reviewed in Jonsson and Hubscher, 1997, Kelman, 1997). These proteins exist as stable trimers that form a closed ring with a hole in the center where the duplex DNA can slide through. It functions as a processivity factor for pol δ during DNA replication. PCNA can be loaded onto the DNA by RFC in an ATP-dependent manner (Tsurimoto and Stillman, 1991). The variety of interactions of PCNA with other DNA metabolism proteins suggests that PCNA is a central factor for the coordination of DNA replication, DNA repair, epigenetic inheritance, and cell cycle control (Waga and Stillman, 1998).

Enzymes Participating in RNA Primer Removal

Human FEN1 is a single polypeptide of 46 kDa with a 5'→3' exo/endonuclease activity that is required for Okazaki fragment maturation (reviewed in Bambara *et al.*, 1997, Waga and Stillman, 1998). Biochemical studies shown that FEN1 functions specifically to remove the RNA primer attached to the 5' end of each Okazaki fragment (Murante *et al.*, 1992, Turchi and Bambara, 1993). When provided with a flap structure containing a 5' segment of DNA or RNA that is not paired to template DNA, FEN1 efficiently cleaves at the branch point, releasing the unpaired segment (Harrington and Lieber, 1994). An Okazaki fragment that is completely annealed to a single-stranded DNA cannot be degraded by FEN1 alone but a ribonuclease in addition to FEN1 is required (Turchi *et al.*, 1994).

As with FEN1, RNase HI is thought to be involved in the removal of RNA primers during Okazaki fragment synthesis (Turchi *et al.*, 1994). It endonucleolyticaly cleaves RNA that is attached to the 5' end of a DNA strand, such as in an Okazaki fragment, leaving a single ribonucleotide on the 5' end of the DNA strand (Turchi *et al.*, 1994, reviewed in Waga and Stillman, 1998)

DNA Helicase

DNA helicases promote the processive unwinding of duplex DNA. This unwinding needs to occur at the DNA replication fork to create templates for the polymerases (reviewed in Waga and Stillman, 1998). SV 40 large T antigen functions as the replicative DNA helicase in the *in vitro* replication, but the helicase involved in cell chromosome replication is still not defined, although several mammalian helicases are identified to be involved in replication (reviewed in Waga and Stillman, 1998). Mouse helicase B is suggested to be involved in a process during replication that does not determine the elongation rate of the fork (Matsumoto

et al., 1995). The proteins that most resembles SV 40 Large T antigen in its structure are the mini-chromosome-maintanance proteins. These proteins are essential components of the prereplication complex established prior to S-phase at origins of DNA replications (Kubota *et al.*, 1995, Todorov *et al.*, 1995). This protein complex is suggested to be a hexamer containing equal amounts of each of the six proteins, typical of the replicative helicases from other organisms, and it was observed to function as a replicative DNA helicase at the cellular replication fork (reviewed in Waga and Stillman, 1998, Tye, 1999).

DNA Ligase

DNA ligase I has been shown to participate in SV 40 DNA replication *in vitro* for the maturation of daughter DNA molecules into closed circular form I DNA (Waga and Stillman, 1994). Human cells with defects in DNA ligase I indicated the enzyme involvement in the *in vi*vo DNA replication process (Henderson *et al.*, 1985, Barnes *et al.*, 1992). It is suggested that DNA ligase I functions to join the Okazaki fragments during replication.

Synthesis of Leading and Lagging Strand

Li and Kelly (1984) developed the first mammalian-based DNA replication system that successfully initiated DNA synthesis *in vitro*. This system requires two viral components, the viral replication origin DNA sequence and SV 40 large T antigen. T antigen recognizes and binds to the viral replication origin, and as mentioned before it also has a helicase activity that melts the DNA in the replication origins (Fanning and Knippers, 1992). The *in vitro* SV 40 DNA replication system has been tremendously beneficial in understanding of the processes taking place in

leading and lagging strand DNA synthesis in mammalian cells (reviewed in Hickey and Malkas, 1997, Burgers, 1998, Bambara *et al.*, 1997, Waga and Stillman, 1998).

Synthesis of the leading strand involves the viral protein large T antigen binding the viral DNA sequence origin and utilizing a 3'→5' helicase activity to separate the strands to create two replication forks (Li and Kelly, 1984). Unwinding of the origin by SV 40 large T antigen is stimulated by the replication protein A (RPA) (Wobbe *et al.*, 1987, Wold and Kelly, 1988). After unwinding, each leading strand is primed by RNA primers generated by the primase subunits of DNA pol α (Wang, 1991). The polymerase subunit of DNA pol α then adds a stretch of deoxyribonucleotides to the RNA primer. Replication factor C (RFC) then initiates a reaction which is called polymerase switching (Waga and Stillman, 1994). RFC dissociates from DNA pol α-primase complex in an ATP-dependent process and assembles proliferating cell nuclear antigen (PCNA) in the region of the primer terminus.

In the *in vitro* SV 40 replication system, priming by DNA pol α–primase and then the switching to DNA polymerase δ occur similarly on both the lagging and leading strands during DNA synthesis (Waga *et al.*, 1994a). However, synthesis of lagging strand requires a lot of polymerase switching (Malkas, 1998). Nevertheless, polymerase switching was found to be a necessary prerequisite for complete gap filling in the lagging strand synthesis (Waga *et al.*, 1994a). Okazaki fragment intermediates consist of RNA primer averaging about 10 nucleotides in length, which was extended with 10-20 additional deoxyribonucleotides (Nethanel *et al.*, 1992). Because Okazaki fragment intermediates are made in the absence of ATP, and RFC requires ATP for polymerase switching, it was thought that the 10 –20 additional deoxyribonucleotides in the intermediates were added by DNA pol α-primase before the switch (reviewed in Bambara *et al.*, 1997). An additional 10-20 nucleotides are added prior to the position of the next downstream RNA primer after loading of PCNA and DNA pol δ (polymerase switching). It is thought that there

would be further extension of the upstream primer during or after removal of the initiator RNA and possibly nick-translation synthesis through the first deoxyribonucleotides of the downstream primer (reviewed in Bambara *et al.*, 1997). RNase H1 then endonucleolytically cleaves the initiator RNA one nucleotide upstream of the RNA-DNA junction (Turchi *et al.*, 1994). The remaining ribonucleotide is removed by FEN1/RTH1 (Waga *et al.*, 1994a, Turchi *et al.*, 1994). The joining of adjacent Okazaki fragments to complete lagging strand synthesis is mediated by DNA ligase I (Turchi and Bambara, 1993).

DNA Replication Factories

In eukaryotes, DNA synthesis appears to occur within replication foci (reviewed in Jackson, 1995). Each replication foci contain many replicons, and within them DNA synthesis is mediated by a single mega complex of proteins (Hozak *et al.*, 1993). Multiprotein replication complex has been purified, and this multicomplex was fully competent to replicate DNA *in vitro* (Malkas *et al.*, 1990, Applegreen *et al.*, 1995, reviewed in Malkas, 1998). The human multiprotein replication complex can be analyzed by sucrose gradient analysis and was observed to have a sedimentation coefficient of 18 S.

Replication foci assemble in cells in a cell-cycle-dependent manner; they initially appear in the late G1 phase and are maintained throughout S-phase (Hozak *et al.*, 1994). There are defined characteristic patterns of DNA synthesis during S-phase that suggest a specific S-phase program for replication (Hozak *et al.*, 1994, reviewed in Jackson, 1995, Malkas, 1998). Temporally coordinated activation of different sets of replicons occurs throughout S-phase, and there is evidence that the sites are influenced by nuclear structure. These replication factories also appear to be associated with the nuclear matrix (Hozak *et al.*, 1993).

DNA Replication and the Cell Cycle

DNA replication occurs only once per cell cycle. The regulation of DNA replication is mediated by a variety of proteins including cyclin proteins, cyclin-dependent protein kinases (cdks), cyclin dependent protein kinase inhibitors (CDIs), oncogene and tumor suppressor gene products, and some transcription factors that are thought to enhance the utilization of replication origins (reviewed in Hickey and Malkas, 1997). An overview of how DNA replication is regulated in the cell-cycle is summarized below.

Cyclin D associated with either cdk4 or cdk 6 (Matsushime *et al.*, 1992, Meyerson and Harlow, 1994) and cyclin E associated with cdk2 (Dulic *et al.*, 1992, Koff *et al.*, 1992) are thought to regulate cells' progression from G1 to S-phase. Cyclin D-cdk4 complex regulates the passage of cells through START, the G1 checkpoint that commits cells to DNA synthesis (Matsushime *et al.*, 1992, Geng and Weinberg, 1993). Several interactions of cyclin D with other proteins modulate its activity, which in turn would regulate cell-cycle progression. p27 tumor suppressor gene expression can inhibit the cyclinD-cdk4 kinase activity and prevent cells from progressing into S-phase and inititating a new round of DNA synthesis (Toyoshima and Hunter, 1994). Transcription factor TGF-β is also known to reduce the cyclin D-cdk4 complex activity (Toyoshima and Hunter, 1994). Interaction of the retinoblastoma (Rb) and the c-myc proteins with the gene encoding cyclin D1 upregulates cyclin D expression (Muller *et al.*, 1994, Marhin *et al.*, 1996). *In vivo,* it has been observed that cyclin D-cdk4 phosphorylates Rb (Horton *et al.*, 1995, Connell-Crowley *et al.*, 1997). In G1 cells, the activation of transcription factor E2F from the Rb-E2F complex can be conferred by cyclin D-cdk4-dependent phosphorylation of Rb. Inactivation of Rb by phosphorylation enables cells to progress into S-phase and initiate DNA synthesis (Wu *et al.*, 1996, reviewed in Weinberg, 1995).

Another cyclin, cyclin E, is required to complete cell progression through G1 and enables the cells to traverse the G1/S-phase border (reviewed in Heichman and Roberts, 1994, Ohtsubo *et al.*, 1995). The cyclin E-cdk2 complex is essential for progression into S-phase since it is known to stimulate the transcription of genes that are required for S-phase (Lees *et al.*, 1992). The cyclin-dependent kinase inhibitor p27 can inhibit the cells from crossing through the G1/S border by binding to the cyclin E-cdk2 (Polyak *et al.*, 1994, Slingerland *et al.*, 1994).

In S-phase, cyclin A is essential for progression through the phase, and it is also required in the molecular processes required to support progression of cells through the G2/M border (Pagano *et al.*, 1992). Cyclin A-cdk2 complex is thought to down-regulate the genes that are required for G1/S progression which were activated by cyclin E-cdk2 in early S-phase, through transcriptional inactivation (Dynlacht *et al.*, 1994, Krek *et al.*, 1994). Cyclin A is degraded following G2/M transitions (Wang *et al.*, 1992).

Cyclin B interacts with a cdc2-related kinase to form mitosis-promoting factor (MPF) that drives the cells through mitosis, and subsequently enables cells to form preinitiation complexes (Adachi and Laemmli, 1994). There is evidence to suggest that cells become competent to replicate DNA during the M phase of the cell cycle (Adachi and Laemmli, 1994), although the cells do not commit to DNA synthesis until they have progressed through M and START (Roberts, 1993). Cyclin B-cdc2 complex is maintained at the inactive state through phosphorylation of Tyr-15 and Thr-14 on the cdc2-related kinase molecule (reviewed in King *et al.*, 1994, Draetta *et al.*, 1988). The kinase is activated by dephosphorylation carried-out by Cdc25 phosphatase at the end of G2 phase (Gautier *et al.*, 1991). At the end of M-phase cyclin B-cdc2 complex is proteolytically inactivated to allow the cells to progress into the G1 phase (Murray *et al.*, 1989, Gallant and Nigg, 1992).

Secondary levels of control for cell cycle progression are through a series of proteins known as cyclin-dependent kinase inhibitors (CDIs) that are thought to bind directly to the cyclin-cdk complexes and inhibit their activity (reviewed in Hickey

and Malkas, 1997). Some of these CDIs are p15, p16, p21 and p27. p21 is known to bind and inhibit the activity of cyclin D-cdk4, cyclin E-cdk2, cyclin A-cdk2 and PCNA (Xiong *et al.*, 1993, Waga *et al.*, 1994b). p21 binding to PCNA inhibits the PCNA-mediated stimulation of DNA polymerase δ activity is evidence of a direct regulation of DNA replication machinery by a cell cycle-related protein. p21 expression is induced by p53, which is known to be induced in response to DNA damage (El-Deiry *et al.*, 1993). This observation correlates with other observations showing that DNA damage can arrest cells during S-phase. P27, another CDI, is capable to prevent the initiation of DNA synthesis by binding and inhibiting cylin E-cdk2 with the net effect of preventing cells from crossing the G1/S border (Polyak *et al.*, 1994, Slingerland *et al.*, 1994).

DNA Replication Fidelity

The high fidelity of DNA replication is ensured by three main factors: the high intrinsic accuracy of DNA polymerases in inserting and selecting for correct template-dNTP base pairing, the exonucleolytic proofreading activity associated with the replicative polymerases and the actions of mismatch repair proteins that correct mismatches in the newly replicated DNA (reviewed in Kunkel and Bebenek, 1988).

The underlying principle of polymerases selectivity in discriminating for correct base pairing of template and incoming dNTP is the precise geometry of Watson-Crick base pair (reviewed in Echols and Goodman, 1991). Other than the A-T and G-C base pairs, all other base pairing combinations have altered geometries. It is thought that DNA polymerase active site prefers the correctly base paired Watson-Crick geometry over the non-Watson and Crick base pairs such as wobble base-pair structures. However, the free energy differences of matched versus mismatched base pairs in aqueous solution could not account for the insertion accuracy observed for most DNA polymerases, which is in the range of 10^3 to 10^5, since the free energy

differences are only approximately ten-fold. It has been suggested that the hydrogen-bonded water molecules are excluded from the transition state for complementary base pairs within the active site, and the active site exclusion of water may increase the base pair free energy (Kunkel, 1992b). Other factors have been suggested to affect nucleotide discriminations. These include base stacking and specific amino acid interactions with the DNA template-primer in the polymerase active site (reviewed in Echols and Goodman, 1991, Goodman, 1997).

Polymerization of a single nucleotide can be divided into several steps as shown in Figure 1.2 (taken from Johnson, 1993). The kinetic mechanism for DNA polymerization has been derived mainly from prokaryotic polymerases DNA pol I (Klenow Fragment) and T7 DNA polymerase (reviewed in Kunkel, 1992b, Johnson, 1993). More recent kinetic studies on mammalian DNA polymerases have shown that the mechanism of single base pair polymerization in mammalian pol δ and γ follows similar mechanism to that of the prokaryotic DNA polymerases (Einolf and Gengerich, 2000, Graves *et al.*, 1998). From kinetic data, it is suggested that nucleotide selectivity of polymerization is derived from several stages in the reaction cycle. In T7 DNA polymerase, correct dNTP is bound with much higher affinity than incorrect dNTP (Patel *et al.*, 1991). The selectivity, which is defined as the ratio of k_{cat}/K_M of correct versus incorrect polymerization, is about 10,000 (Patel *et al.*, 1991). Polymerization is much slower for incorrect dNTP than for correct dNTP (Patel *et al.*, 1991). Selectivity is conferred after dNTP binding but preceding the phosphodiester bond formation (Patel *et al.*, 1991). It is thought to involve enzyme-dNTP conformational changes that positions dNTP for catalysis termed induced fit selectivity (reviewed in Johnson, 1993). In the case of incorrect dNTP binding to the enzyme-DNA complex, slower phosphodiester bond formation can be observed (Wong *et al.*, 1991). Among DNA polymerases that have been studied there are variations in the actual extent of discrimination for each of these steps.

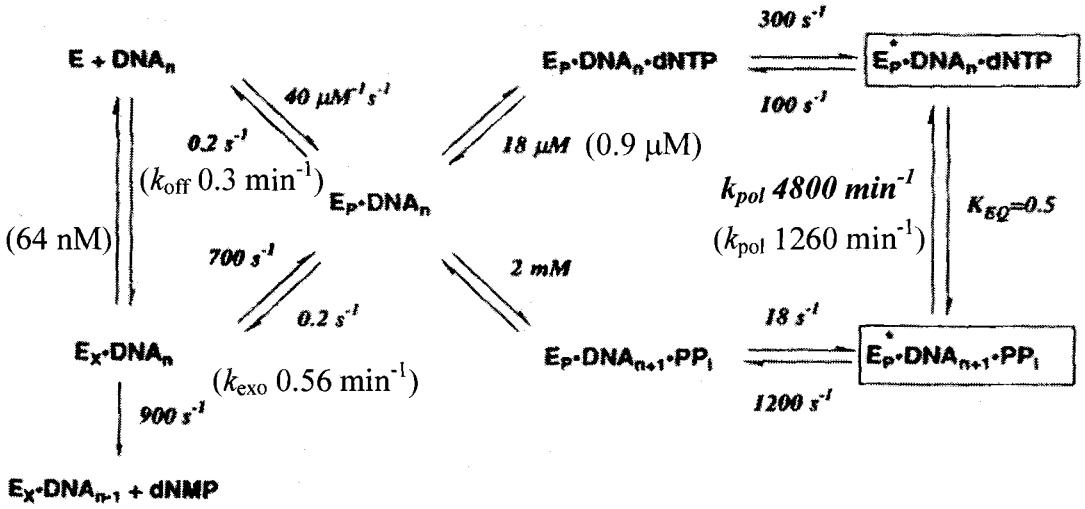

Figure 1.2. Kinetic Mechanisms for DNA Polymerization. The values in bold are obtained from T7 DNA polymerase (adapted from Johnson, 1993). Kinetic parameters inside parentheses are from fetal calf thymus DNA polymerase δ in the presence of PCNA (Einolf and Guengerich, 2000).

DNA pol δ polymerize DNA at a slower rate than T7 DNA polymerase (as shown in Fig. 1.2). Pol δ has much lower Kd for dNTP, and in the presence of PCNA, the DNA does not dissociate from pol δ as often as in case of T7 DNA polymerase. Moreover, it is believed that different mispairs can also modulate the selectivity of DNA polymerases (reviewed in Johnson, 1993, Kunkel and Bebenek, 2000).

Proofreading functions add an additional 100-fold to the fidelity of DNA polymerases. This number is obtained from studies of polymerases in which the proofreading activity has been compromised either by addition of nucleoside monophosphate, or a high concentration of dNTP, or site-directed mutagenesis of a residue that is essential for proofreading activity (reviewed in Kunkel, 1992b). The kinetics of DNA misincorporation comes largely from studies with exo$^-$ T7 DNA polymerase (Wong *et al.*, 1991) and *E. coli* pol I Klenow fragment (Eger and Benkovic, 1992, Dahlberg and Benkovic, 1991). In Figure 1.3 it is shown that the rate of insertion and polymerization from a correctly paired terminus is faster

compared to the dissociation rate or excision from a correct terminus. However, when misincorporaton has occurred, the rate of mismatch extension is slow while the pyrophosphorolysis rate is negligible.

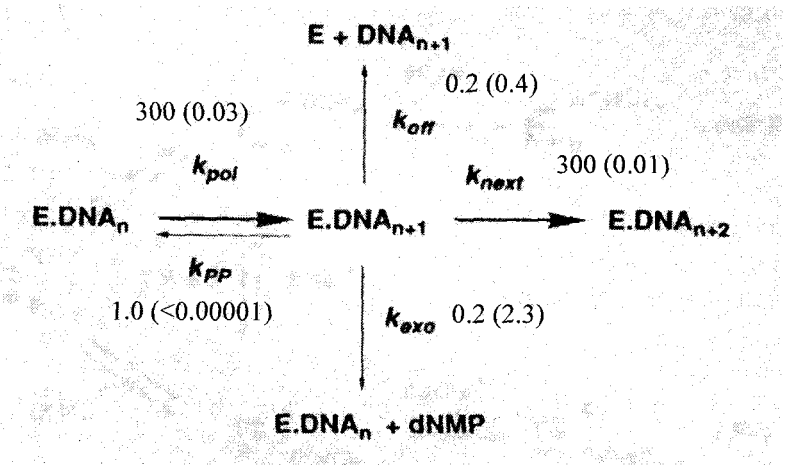

Figure 1.3. Kinetics of Exonuclease Proofreading. The kinetic constants are from Patel *et al.* (1991), Donlin *et al.* (1991) and Wong *et al.* (1991) for T7 DNA polymerase. The values and kinetic scheme were summarized previously (Johnson, 1993).

In DNA pol I (Klenow fragment), a second conformational change after formation of phosphodiester bond is thought to follow misincorporation (Dahlberg and Benkovic, 1991) that was not observed for T7 DNA polymerase (Wong *et al.*, 1991). The mechanistic difference could be explained by the functional difference between the two enzymes. DNA pol I (Klenow fragment) functions as repair polymerase instead of replicative polymerase like the T7 polymerase. It was also shown that exonuclease activity of mismatched termini may be enhanced significantly by instabilities of local primer termini (Bloom *et al.*, 1994). In T7 DNA polymerase, the excision of mismatched termini occurs faster than matched nucleotide-DNA termini with rate-limiting translocation of terminal nucleotides to exonuclease active site (Figure 1.3). The fast rate of translocation back to

polymerase site prevents the exonuclease activity from removing additional nucleotides from the termini (reviewed in Johnson, 1993, Kunkel and Bebenek, 2000). It has been observed that rates of extension from different mismatches are varied (Perrino and Loeb, 1989, Creighton *et al.*, 1992). Different mismatch termini probably modulate the exonuclease proofreading efficiency to some degree.

Mismatch repair associated with newly replicated DNA contributes to DNA replication fidelity, as not all mismatches are corrected by exonucleolytic proofreading. Some mispairs, such as G/T wobble pair, do not distort the helix significantly and are known to be able to be extended by polymerases (reviewed in Jiricny, 1998). Other errors that might not be corrected by exonuclease proofreading are loops created by primer-template misalignments. These loops are the substrate for mismatch repair (Jiricny, 1998). Mismatch recognition is the initial step for repair. hMutSα, composed of hMSH2/6 complex, is thought to preferentially bind base-base mismatches and loops of one nucleotide (Palombo *et al.*, 1995, Palombo *et al.*, 1996, Acharya *et al.*, 1996), and another complex, hMutSβ, which is composed of hMSH2/3, is thought to recognize other loop structures (Palombo *et al.*, 1996, Acharya *et al.*, 1996). hMLH1/hPMS2 and hPMS1 are the human homologs of the *E. coli* MutL protein (Li and Modrich, 1995). hMLH1/hPMS2 (hMutLα) complex is found to interact with hMutS complexes that is bound to the mismatched or looped DNA (Matton *et al.*, 2000). No biochemical role has been defined for the third human homolog of *E. coli* MutL, hPMS1 protein, although there is evidence for interaction between hMLH1 and hPMS1 to form hMutLβ complex (Raschle *et al.*, 1999). Other proteins that are thought to participate in mismatch repair processes are PCNA and pol δ as well as the single-stranded DNA binding protein, RPA (reviewed in Jiricny, 1998, Kolodner, 1995).

DEOXYRIBONUCLEOSIDE TRIPHOSPHATES AND MUTAGENESIS

dNTP imbalance, produced in cells through a variety of means, such as exposure to mutagens, administration of drugs that disrupt the enzymes involved in dNTP metabolism, mutations affecting dNTP metabolic enzymes, or addition of bases or nucleosides to the growth medium, has been widely documented to have various effect on the cells' mutation frequency (reviewed in Kunz and Kohalmi, 1991, Kunz *et al.*, 1994, Meuth, 1989). In some cells specific dNTP perturbations change the observed mutation frequency, while in other cells no effects on mutation frequency were observed (reviewed in Kunz and Kohalmi, 1991, Kunz *et al.*, 1994, Meuth, 1989).

In vivo, the effects of dNTP imbalance have been studied through sequencing of mutants. Mechanisms of mutations causing dNTP imbalance are thought to occur through misinsertions of the dNTP that is higher in concentration compared to other dNTPs during replication and through the "next-nucleotide effect" (Kunkel, 1988), which is the term used for the observed preference of misincorporation when the next nucleotide is present at high concentration. It is thought that in a situation where excess nucleotide is present during replication, two mechanisms leading to next-nucleotide effect can be envisioned. First, increase in nucleotide concentrations can increase the probability of extending a mismatched terminus, and second, enhanced polymerization rates can reduce the probability that a mismatched terminus is corrected by the exonuclease proofreading. Both effects of increased nucleotide concentrations, increase in substitution mutation and next-nucleotide concentration, were observed *in vivo*; however, there are some base pair changes that sometimes predominates among mutants sequenced but are not explained by the two effects (reviewed in Kunz and Kohalmi, 1991). Other mechanisms that can also generate mutations in condition of dNTP imbalance involved template-primer misalignment (Kunkel, 1990). Most of the resulting mutations involving this mechanism would

result in frameshift mutations. It is not evident to what extent template-primer misalignment contributes to mutations induced by imbalanced dNTP *in vivo*.

OBJECTIVES

The main focus of this thesis is to explore the relationship between dNTP levels during replication and the observed spontaneous mutation frequency *in vitro* and *in vivo*. Specifically, three questions involving dNTP levels and DNA replication fidelity will be addressed:

1. What is the effect of dGTP underrepresentation on replication fidelity in mammalian cells *in vitro*? What is the effect of different dNTP pool levels obtained from immortal and normal diploid cells lines on the observed mutation frequency *in vitro*?
2. Is there a modulation of dNTP levels during S-phase in mammalian cells *in vivo*? Is there a difference in the rates of DNA replication at different times during S-phase in mammalian cells?
3. Is there a difference in the *in vivo* spontaneous mutation rates between genes that are replicated at different time during S-phase in mammalian cells? If there are differences, do they correspond to differences in dNTP levels or DNA replication rates during S-phase?

The first question, which will be addressed in Chapter 2, deals mostly with the observations of underrepresentation of dGTP in almost all cell lines studied (reviewed in Mathews and Ji, 1992). However, the dNTP pool measurements obtained were mostly from immortal or somatic cell lines; therefore, studies on the effect of different dNTP pools measured from immortal cell lines derived from cancer cells and normal diploid cell lines derived from somatic cells on replication fidelity *in vitro* were also done. Most mammalian tissues are composed of resting cells not directly relevant to this *in vitro* study. These resting cells have negligible *de*

novo nucleotide synthesis (Arner and Eriksson, 1995) and dNTP pools. However, it has been documented that in cancer cells nucleotide synthesis enzymes, such as thymidine kinase, are present at higher levels than their levels in cycling somatic cells (Gordon *et al.*, 1968). Regulation of enzymes involved in nucleotide synthesis has also been reported to be altered in cancer cells (Hengstschlager *et al.*, 1994, Hengstschlager *et al.*, 1996). These could provide the basis for the different levels of dNTP pools between the two kinds of cell lines. Whether the difference in dNTP pool levels extracted from the immortal and normal diploid cell lines can modulate DNA replication fidelity *in vitro* is evaluated in the study.

It has been proposed that different ratios of dNTP pools during S-phase in germ line cells could account for the existence of isochores (Wolfe *et al.*, 1989), approximately 300-kb regions in mammalian chromosomes that have similar G+C contents in DNA base pair composition (reviewed in Sabeur *et al.*, 1993). The dNTP pool levels of HeLa cells during S-phase were evaluated, as presented in Chapter 3. Concomitant evaluation on DNA synthesis rates at specific times during S-phase were also evaluated to examine the relationship between the measured dNTP pool levels and *in vivo* DNA synthesis rates in HeLa cells.

In Chapter 4, the effect of the time a specific gene is replicated in S-phase is evaluated. Modulation of dNTP pool levels and DNA synthesis rates could affect the mutation frequency *in vitro*; therefore, the effect of replication time during S-phase, which corresponds to modulation of dNTP pools and DNA synthesis rate in HeLa cells, on spontaneous mutation frequency *in vivo* was evaluated.

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CHAPTER 2

EFFECTS OF BIOLOGICAL DNA PRECURSOR POOL ASYMMETRY UPON ACCURACY OF DNA REPLICATION *IN VITRO*

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SUMMARY

Deoxyguanosine triphosphate (dGTP) is underrepresented among the four common deoxyribonucleoside triphosphates (dNTPs), typically accounting for 5 to 10 percent of the total dNTP pool. The research conducted in this section will address whether this pool asymmetry affects the fidelity of DNA replication by use of an *in vitro* assay in which an M13 phagemid containing the *Escherichia coli* lacZα gene and an SV 40 replication origin is replicated by extracts of human cells. By monitoring reversion of either a TGA or a TAA codon within the lacZα gene, different pattern of mispairing events leading to mutations was observed in replication reaction with "biologically biased" dNTPs, representing the estimate of the concentrations in HeLa cell nuclei. However, replication reaction with biased dNTPs is not significantly more accurate than replication reaction containing four dNTPs at equimolar concentrations (100 μM). During replication at biased dNTP levels, mutations at the site 5' to C in the template strand for the TGA triplet had a tendency to be less frequent than seen in equimolar reaction mixtures, suggesting that extension from a mismatch at this site is relatively inefficient, when dGTP is the next nucleotide to be incorporated. Furthermore, an excessively low concentration of one dNTP is not as mutagenic in replication reactions with biased dNTP concentrations as an excessively high concentration of a single dNTP. The dNTP concentrations in non-transformed human fibroblasts were also estimated, and it was found that *in vitro* replication at these levels caused considerably fewer mutations than that observed under equimolar conditions (100 μM each dNTP). Replication rates are decreased somewhat at these non-transformed fibroblast concentrations, inferring an efficient polymerization rate at this lower dNTP concentrations. Replication fidelity is enhanced in replication reactions with dNTPs at non-transformed fibroblast concentrations, presumably from much lower efficiency of

mismatch extensions in this reaction than in replication reactions with higher, although equimolar, dNTP concentrations.

INTRODUCTION

Intracellular concentrations of the four deoxyribonucleoside triphosphates (dNTPs) are normally controlled within narrow limits, through regulation of biosynthetic and degradation rates (reviewed in Reichard, 1988). Perturbations of the "normal" dNTP balance have been documented to have divergent effects in cells, and most of them lead to an increase in spontaneous mutation frequency (reviewed in Kunz and Kohalmi, 1991; Reichard, 1988). However, the specific effect of unbalanced dNTP pools in cells on replication error frequency is not always clear. Perturbations of dNTP pools *in vivo* have been achieved by several means, such as adding nucleobase to increase dNTP pools *via* the salvage pathway or adding drugs to inhibit an enzyme in dNTP metabolism (Meuth, 1989). Also, strains that have a defect in dNTP pool metabolism and therefore have either an increase or decrease in one or more pools as compared to normal cells have been studied to give an insight on the effect of unbalanced dNTP pools on mutation frequency. Since most of the treatments to achieve pool perturbation are not specific to one dNTP, it is difficult to relate the observed effect on DNA replication accuracy (reviewed in Meuth, 1989).

In vitro DNA replication experiments have shown that dNTP concentration is an important determinant of DNA polymerase fidelity (Kunkel, 1992). Increasing the concentration of one nucleotide in the replication reaction can increase mutation frequency significantly. Under certain conditions, decreasing the concentration of one or more nucleotides has also been shown to increase the observed mutation frequency (Roberts and Kunkel, 1988; Perrino and Loeb, 1988; Bebenek *et al.*, 1992).

Given the extent of dNTP pools' effects on replication fidelity, it is quite surprising that whole cell dNTP pool measurements of various mammalian cells in culture are not symmetric. dGTP is almost always underrepresented (reviewed in Mathews and Ji, 1992). dNTP pool measurement from frozen rat embryo also shows that dGTP is lowest in concentration, suggesting that whole cell dNTP pools measured in cultured cells resemble the concentrations found in intact cycling and living cells (Mole *et al.*, 1998). Previously Dr. Mathews' laboratory also has been able to measure the dNTP pool concentrations of HeLa cell nuclei, and they approximate the molarity of the whole cell pool measurements (Leeds *et al.*, 1985).

Measurement of dNTP pools in cells repesents the level of the cells' steady state dNTP concentrations as the result of the difference between the rate of their production and use or degradation. Therefore, it is questioned whether dNTP pool measurement resembles the actual dNTP concentrations available at the replication fork. One issue that has been put forth is the possibility of compartementation of pools in eukaryotic cells. However, experiments that addressed dCTP pool compartementation showed different results in the different cell lines (Xu *et al.*, 1995, Nicander and Reichard, 1983). Moreover, *in vivo* experiments (Meuth, 1989) showed a clear indication that an increase in the measured dNTP pools produced the expected kinds of substitution mutations, suggesting similar increase in the specific pools at the replication fork. Based on Meuth's observations, the experiments presented here were based on the premise that the measured nuclear dNTP pools probably represent a good approximation of the dNTP concentrations available at the replication forks in eukaryotic cells.

To test the effects of biologically biased dNTP concentrations of HeLa cell nuclei on DNA replication accuracy, human cell extracts were analyzed in the *in vitro* assay for reversion of an M13 phagemid which had an SV 40 origin of replication in reaction mixtures containing either biologically biased or equimolar dNTP concentrations. Mutation frequency is defined as the number of revertants out of total number of plaques counted. Revertants arise when an incorrectly base-paired

dNTP is inserted opposite a DNA template and not proofread by the exonucleolytic proofreading function associated with human replicative DNA polymerases. The effects of underrepresentation of dGTP on proofreading activity on a mispair 5' to a template C and on the higher probability of misincorporation opposite template C are purposely addressed. These events were predicted to be the events most likely influenced by the small size of the dGTP pool.

MATERIALS AND METHODS

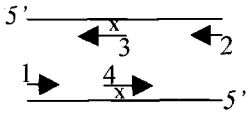
DNA CONSTRUCTS, BACTERIAL STRAINS AND CELL LINES

Mutants of the phagemid M13mp2SV (Roberts and Kunkel, 1993) were used which contained an SV 40 replication origin and the first 45 codons of *Escherichia coli* β-galactosidase with 115 nucleotides of upstream sequence. A derivative of the phagemid, which is designated M13mp2SV/opal7 in this study, contains an opal codon at position 7 of the *lacZα* peptide and has been described before in Zhang and Mathews (1995). Another derivative was made of the M13mp2SV phagemid, M13mp2SV/ochre7, which was constructed by site directed mutagenesis of the wild type M13mp2SV using PCR (Ho *et al.*, 1989). This construct contains an ochre codon at position 7 and an AAC codon, which is a silent mutation at position 6. Below is the sequence of the relevant region of M13mp2SV plasmid and its derivatives, and introduced mutations are shown in bold.

M13mp2SV/ochre7 was constructed by site-directed mutagenesis using PCR (Ho *et al.*, 1989). PCR primers used in the site directed mutagenesis were: MPA (1), $^{5'}$*GGA CTA TGG TTG CTG AC*; MPB (2), $^{5'}$*GAA GAT CGC ACT CCA GC*;

	codon number of *lacZα*						
	4	5	6	7	8	9	10
M13mp2SV	ATT	ACG	AAT	**TCA**	CTG	GCC	GTC
M13mp2SV/opal7	ATT	ACG	AAT	**TGA**	CTG	GCC	GTC
M13mp2SV/ochre7	ATT	ACG	AA**C**	**TAA**	CTG	GCC	GTC

MPC (3), $^{5'}$*GGC CAG TTA GTT* CGT *AAT CAT G* and MPD (4), $^{5'}$*GAT TAC GAA CTA ACT GGC CGT C*. Primers were used as diagrammed :



The first sets of amplification were with primers 1 and 3 and also primers 2 and 4 with the M13mp2SV DNA template. The second set of amplification was with primers 1 and 2 and the DNA fragments generated from the first sets of amplification were used as template. The resulting gene fragment was digested and ligated back into M13mp2SV phagemid.

Eschericia coli CSH50, NR9162 and NR9099 were obtained from Dr. Thomas Kunkel (NIEHS). *E. coli* NR11089 was kindly constructed by Dr. Roel Schaaper (NIEHS). This strain is isogenic to CSH50 with an exception of having an ochre suppressor at amino acid position 7 of LacZα. M13mp2SV/ochre7 gave a medium blue phenotype when plated on *E. coli* NR11089. Prolonged incubation (30 hours or more) of M13mp2SV/opal7 phagemid plated on *E coli* NR11089 usually results in a very light blue phenotype, which suggests a slow leaky phenotype of the ochre suppressor for the opal codon at the same position. HeLa S3 and HCT 116 cell lines were obtained from American Type Culture Collection (ATCC). HCT 116 cells

are derived from a colon tumor and are defective in mismatch repair. HeLa S3 cells were grown in S-MEM medium (Gibco BRL) supplemented with 5% fetal bovine serum. HeLa cells were grown in suspension, and 1 L of cells was used for preparation of extracts as described by Roberts and Kunkel (1993). HCT 116 cells were grown in McCoy 5A medium supplemented with 10% fetal bovine serum. The cellular contents of 40 150-mm plates were trypsinized and combined for preparation of extracts.

REPLICATION REACTION

Preparation of Cellular Extracts

The human cell extracts preparation was essentially as described by Roberts and Kunkel (1993). For extraction of spinner culture cells, 1 L of cells was harvested at mid-log growing phase. The cells were pelleted at 1,500 rpm for 5 min at 4°C in a Beckman table-top centrifuge. The pellet was washed twice in ice-cold phosphate-buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L double-distilled H2O (ddH_2O), prepared as 10x solution), followed by one wash in cold isotonic buffer (20 mM HEPES-KOH (pH 7.8), 5 mM KCl, 1.5 mM $MgCl_2.H_2O$, 250 mM sucrose and 1 mM DTT that was added just before use) and one more wash of cold hypotonic buffer (20 mM HEPES-KOH (pH 7.8), 5 mM KCl, 1.5 mM $MgCl_2.H_2O$, 1 mM DTT) before finally resuspending the pellet in a total volume of 5 mL of hypotonic buffer. The cell suspension was transferred to a 7-mL Dounce homogenizer (Kontes) on ice. The cells were allowed to swell in the hypotonic buffer on ice for 60 minutes.

For extraction of HCT 116 cells, which were grown in plates, 40 150-mm plates of log-phase growing HCT 116 cells were pooled. Each plate was trypsinized,

and the cells were pooled into 4 separate 50-mL conical centrifuge tubes. The cells were pelleted and washed as described above for cells grown in spinner culture. The four tubes were pooled into one 50-mL conical centrifuge tubes for the hypotonic buffer wash and resuspension. As with spinner cell extraction, the cells then were transferred to the Dounce homogenizer in which they were allowed to swell in the hypotonic buffer.

The next steps of extraction were identical for both the spinner and attached cell cultures. The cells were lysed with two strokes of the tight fitting homogenizer, and the extent of cell breakage was determined by adding 50 μL of trypan blue dye solution (0.4% w/v, 1 M $NaHCO_3$, filter sterilized) to 100 μL of cell suspension from the Dounce homogenizer that were then examined using the light microscope. Lysed cells would incorporate the trypan blue dye and would appear blue using the microscope. Usually, four to six strokes were required to cause about 80% cell breakage for HeLa cells, and ten to twelve strokes were required for HCT 116 cells.

The extracts then were transferred to a cold Corex centrifuge tube and centrifugation was conducted at 3,000 rpm in a fixed-angle Sorvall SS 34 rotor for 10 minutes at 4°C to pellet the nucleus and the unbroken cells. The supernatant fraction was transferred to another cold centrifuge tube and centrifugaion was performed at 12,000 rpm in the same rotor at 4°C. The cleared supernatant fraction was transferred to a clean tube, mixed and 10 μl was analyzed to determine the protein concentration using the Bradford method. The rest of the extracts were aliquoted into 500-μL Eppendorf tubes on ice that were immediately dropped into liquid nitrogen and stored in –80°C. The extract usually had a protein concentration of 10-20 mg/mL as determined by Bradford assay. Each aliquot was thawed and centrifuged immediately before use at 14,000 rpm for 15 minutes in a fixed-angle Eppendorf centrifuge at 4°C. The cleared supernatant was transferred to a clean Eppendorf tube and then was ready for use in the replication reaction.

"Dialysed" HeLa cell extract was prepared by processing the thawed and centrifuged extract through the clear Centri-sep column (Princeton Separation)

according to manufacturer's instruction, and then through Microcon 3 centrifugal separation (Millipore) at 10,000 rpm in a fixed-angle Eppendorf centrifuge for 10 – 15 minutes at 4°C immediately before the reaction.

In Vitro Replication Reaction

In vitro replication reactions using either HeLa or HCT 116 cell extracts were essentially as described in Roberts and Kunkel (1993). SV 40 Large T antigen was obtained from Molecular Biology Resources (MBR). Radioactive [α-^{32}P]dATP or dCTP tracer (~1000 cpm/pmol) was used only in confirming the replication activity of the extracts preparation. No radioactive tracer was used in the actual experiments where phages were plated to obtain mutation frequency. The replication reaction (25 μL) contains 30 mM HEPES-KOH (pH 7.8); 7 mM $MgCl_2$; 4 mM ATP; 200 μM each of CTP, GTP and UTP; dNTP (equimolar reactions contain 100 μM of each dNTP and biologically biased dNTP concentrations refer to calculated HeLa cells' nuclei concentrations as reported by Leeds *et al.* (1985), which are 60 μM dATP, 60 μM dTTP, 30 μM dCTP and 10 μM dGTP); 40 mM creatine phosphate (Sigma); 100 mg creatine phosphokinase (Sigma)/mL; 40 ng of replicative form of the template DNA; ~1 μg SV40 T antigen; and ~ 75 μg protein of the extract. It is important to use template DNA that has been purified through a CsCl/ethidium bromide gradient following isolation by anion exchange chromatography (Qiagen). The reaction mixture was incubated at 37°C for ~ 4 hours, and 25 μL of stop solution (2 mg proteinase K/mL, 2% (w/v) sodium dodecyl sulfate (SDS), 50 mM EDTA; the solution was made immediately before use) was added to the reaction which was then incubated again at 37°C for 30 minutes. For determination of ^{32}P incorporation, 10 μL from each reaction was spotted onto Whatman filter paper that had been saturated with 5% trichloroacetic acid (TCA)/0.1 M $Na_2H_2P_2O_7$, and the paper was

washed in 5% TCA/0.1 M $Na_2H_2P_2O_7$ twice for 15 minutes each at room temperature and with 95% ethanol (EtOH) twice for 15 minutes each at room temperature. The dried filter paper was then put into the scintillation vial with ~ 2 mL of scintillation coctail and counted. Otherwise the rest of the reaction mixture was collected by adding 28 μL of precipitation mix (final concentration of 20 μg carrier tRNA and 1 M ammonium acetate) and 100 μL of isopropanol. The DNA was precipitated by incubating at –20°C for at least 20 minutes to overnight. The DNA was collected through centrifugation, and purified through extraction with PEB (100 mM Tris-HCl (pH 8.0), 1 mM EDTA, 300 mM NaCl)-equilibrated phenol (2 x 100 μL). The DNA was precipitated again and collected through centrifugation. It was then digested with excess *Dpn I* (NEB Biolabs) that was tested each time to be active by digesting 40 ng of template DNA alone and by electroporation and plating of the *DpnI* digested DNA control onto the plating *E. coli*. Typically, 40 ng of template DNA digested with *Dpn I* yielded a total of ~10,000 plaques which contribute to less than 0.5% of the total plaques typically yielded in a replication reaction. Products of replication reaction with [α-^{32}P]dNTP tracer can then be characterized by restriction digest analysis (*Dpn I* or *Mbo I*), electrophoresed on 1% agarose-0.5x TBE (0.089 M Tris-borate, 0.002 M EDTA pH 8.0 made as 5x stock solution) gel for approximately 1-2 hours, following which the gel was dried and autoradiographed. The digested DNA to be plated was precipitated again and collected through centrifugation, dried and resuspended in TE buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA).

Plating of the phages was done by electroporating the DNA into ~0.15 x 10^{10} *E. coli* NR9162 (*mutS*). The electroporatant (the calculated amount of DNA electroporated into *E. coli* NR9162 and resuspended in 1 mL of LB (10 g tryptone, 5 g yeast extract, 10 g NaCl in 1 L of ddH_2O, autoclaved)) was plated simultaneously with *E. coli* CSH50 or NR11089 cells that would produce a lawn of α-complementation indicator cells. Minimal media plate (25 mL/plate, 100-mm plates; 1 L of minimal media plate was made up of 15 g Bacto agar in 800 mL H_2O,

autoclaved; 200 mL of 5x salt (1 g $MgSO_4.7H_2O$, 10 g citric acid (anhydrate), 50 g K_2HPO_4, 7.5 g $Na_2HPO_4.2H_2O$, in 1 L H_2O, autoclaved); 20 mL of 20% (w/v) glucose, filter sterilized; and 0.5 mL of 10 mg/mL thiamine hydrochloride, filter sterilized) was used with 2.5 mL of prewarmed (45°C) soft agar (0.8% (w/v) agar, 0.9% (w/v) NaCl, autoclaved) aliquoted together with 25 μL of 24 mg isopropyl thiogalactoside (IPTG)/mL H_2O (filter sterilized, store frozen) and 50 μL of 50 mg/mL of 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-Gal) in *N,N*-dimethylformamide (stored at –20°C, avoid light exposure), and ready to be mixed with the electroporatant and 300 μL log-phase growing *E. coli* CSH50 or NR11089. Plating of all the electroporatant (usually onto 50-60 minimal media plates) was completed within 30 minutes after electroporation to avoid the release of phage particles while they were still in solution. Usually the amount of DNA to be plated was calculated to yield about 2000-4000 plaques/plate in order to be able to clearly see the mutant plaque. Plates with very dense plaques usually give an underestimation of the total mutation frequency since not all of the plague estimated from the counting plates (1 to ~ 50 or 100 dilution phage plated to give the total number of plaques observed) are formed when there is more than ~7000 plaques/plates.

Sequencing of the revertants was done by the Center for Gene Research and Biotechnology (CGRB) sequencing facility at Oregon State University. Each blue plaque was diluted into 0.9% NaCl solution and replated twice until isolated blue plaques could be picked from the *E. coli* lawn. The single stranded M13 DNA from each revertant was isolated according to Del Sal *et al.*, (1989) with some modification. Briefly, to 1 mL of supernatant collected of previously infected culture (the phage culture was collected by centrifuging at 14,000 rpm in an Eppendorf microcentrifuge for 10 minutes to remove bacteria), 0.25 mL of 15% (w/v) polyethylene glycol (PEG)/ 2.5 M NaCl was added. The solution was incubated for more than 5 minutes at 4°C and centrifuged at 15,000 rpm in an Eppendorf centrifuge for ~2 minutes. The pellet was then resuspended in 690 μL of ddH_2O, and

100 μL of 1 M Tris-HCl (pH 8.0), 100 μL of 0.5 M EDTA, and 10 μL of 10 mg/mL proteinase K solution were added before the mixture was incubated at 37°C for 30 minutes. Next, 100 μL of 10 x cetyltrimethylammonium bromide (CTAB) was added, and the mixture was incubated further for 10 minutes at 37°C. The sample was centrifuged at 15,000 rpm in an Eppendorf centrifuge for 10 minutes, and the pellet was resuspended in 300 μL of 1.2 M NaCl, 0.75 mL of 100% EtOH was added, and the sample was incubated at –20°C for at least 20 minutes before collecting the DNA by centrifugation. The pellet was washed in 70% EtOH (v/v), dried and resuspended in 10 μL of ddH_2O ready for sequencing. Assignment of leading and lagging strand of replication for predicting mispairing events leading to mutagenesis is as diagrammed in Figure 2.1.

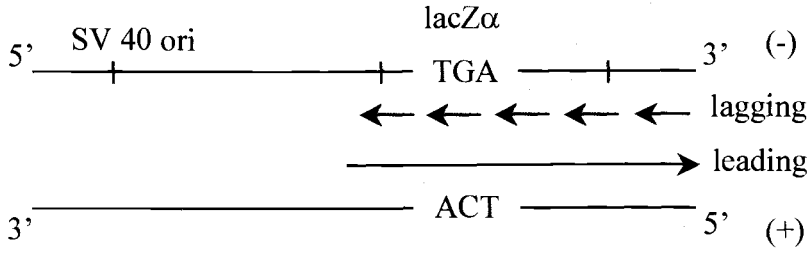


Figure 2.1. Strand Designation for Interpretation of Sequence Results.

dNTP Pool Extraction

HeLa and HCT 116 cell extracts used for the replication reactions were analyzed for their endogenous dNTP contents. To isolate dNTP from the cell extract, 10 μl of ice cold 50% TCA was added to 90 μl of cold cell extract to make final concentration of 5% TCA. The precipitate was immediately centrifuged at 4,000 rpm in the fixed-angle Eppendorf microcentrifuge for 4 minutes then at 10,000 rpm for 5 minutes at 4°C. The supernatant fraction was neutralized with 1.5 volumes of 0.5 M

tri-N-octylamine in Freon (Leeds *et al.*, 1985). After checking that the pH of the upper aqueous phase was around 5.5, the aqueous phase was transferred into a clean microfuge tube and dried in a Speed-Vac (Savant). The residue was redissolved in 100 µl of water or dNTP pool assay buffer (45 mM Tris-HCl, pH 8.3), and it was ready for pool analysis.

dNTP pool assay was done as described previously (Leeds *et al.*, 1985). Essentially, 5 µl of each extracts was added to 45 µl of the reaction mixture to give final concentrations of 45 mM Tris-HCl pH 8.3; 4.5 mM $MgCl_2$; 1 mM β-mercaptoethanol added just before use; 0.2 mg/mL bovine serum albumin (BSA); 8 µg/ml of either poly-dAdT template for quantitating dATP and dTTP or poly-dIdC template for dCTP and dGTP; 1 unit/mL of *E. coli* DNA polymerase I large fragment (Klenow fragment); 10 mM dAMP to inhibit proofreading; 2 µM complementary dNTP and 0.01 µCi/µl of complementary [^{3}H]dNTP. For dATP quantitation dTTP is the complementary dNTP, for dTTP, dATP is the complementary dNTP, for dCTP, dGTP is the complementary dNTP and for dGTP, dCTP is the complementary dNTP. Standards of known amounts of each dNTPs were included in each assay. The amount of dNTP in each unknown samples was determined through interpolation of the standard curve generated for every assay. It is important to note that the use of Klenow exonuclease$^-$ fragment as DNA polymerase in place for Klenow has not worked well for dCTP quantitation as the reactions seemed to be very slow to come to completion. Also, diluting the extracts several-fold seemed to increase the measured dNTP concentration in the extracts. To solve this problem the extracts have been diluted four- to sixteen-fold until no further increase was seen in the values of dNTP concentrations obtained from higher dilutions. The same dilution factor was used for the measurement of all dNTP in the extracts.

Cell lines that were analyzed for dNTP concentrations used in the replication reaction with normal human diploid fibroblast dNTP pools were IMR-90, obtained from ATCC, and NDHF cells obtained from Dr. Janet Leeds (Isis Pharmaceuticals)

in which the pool measurements were done by Linda Wheeler in Dr. C. K. Mathews' laboratory.

RESULTS

EFFECTS OF dGTP BIAS ON MUTATION FREQUENCY

Biologically biased dNTP concentrations can be predicted to have two effects on DNA replication accuracy. First, an increase in mutation frequency opposite template C in DNA can be expected since dGTP is the least abundant in concentration compared to other dNTPs in competing for incorporation. Second, low concentrations of dGTP, that is, below the K_d for correct dNTP binding, could delay its incorporation opposite template C, which would favor exonuclease to act on a mispair prior to template C, hence decreasing mutation frequency 5' to template C. Previous experiments with M13mp2SV/opal7 DNA replicated using HeLa cell extract and either equimolar or biologically biased dNTP concentrations (Zhang and Mathews, 1995) were inconclusive. One reason was the use of HeLa cell extracts with mismatch repair capability that could correct some mispairing errors therefore preventing them from being scored as mutants. Another reason was that one possible error, G→A transition within the opal codon, could not be scored because it yielded an ochre codon which has the same phenotype as the opal codon when plated on *E. coli* CSH50. Therefore, the experiments were repeated with some modifications. Mismatch repair-deficient cell line HCT 116 extracts were used as well as HeLa cell extracts to examine the effect of mammalian mismatch repair complexes on the observed mutation frequency. *E. coli* NR11089, instead of CSH50 as the α-complementing *E. coli,* was used to score all possible revertants of the TGA codon since it carries an ochre suppressor.

As shown in Table 2.1, there is no increase in the mutation frequency differences between equimolar and biased replication reactions when done using HCT 116 compared to HeLa cell extracts. This result agrees with Roberts and Kunkel's (1993) suggestion that the amount of DNA used in the *in vitro* replication reaction is probably sufficiently large to overwhelm the mismatch repair complexes if they are active in the extract. Furthermore, no significant difference in mutation frequencies ($p = 0.281$ Fisher's exact test) was observed between replication reactions with equimolar and biased dNTP concentrations (data used for statistical analysis are presented in Figure 2.2). However, the nature of the data collected suggests that if a small difference were present between the two reaction conditions, the difference would most likely be undetectable due to the large confidence interval for the ratio of the two mutant frequencies (bias over equimolar).

The next question that was addressed was how much of a pool imbalance was needed in order to see a detectable change in the mutation frequency. The dGTP concentration was then systematically lowered while keeping other dNTPs at biologically biased concentrations. In this experiment mismatch repair-deficient cell line HCT 116 extracts were used to replicate M13mp2SV/opal7 DNA in reaction mixtures containing biologically biased dNTPs with high or low dGTP concentrations as shown in Figure 2.2. When dGTP concentration in the replication reaction was systematically lowered, no meaningful increase in mutation frequency was observed even when dGTP concentration was 6000-fold lower than dATP or dTTP in the reaction mixture.

On the other hand, a 100-fold increase in dGTP concentration over the physiological value increased the mutation frequency more than five-fold as was previously shown (Zhang and Mathews, 1995). Shown in Figure 2.2 also is the total number of plaques (pfu) produced in the 25-μl replication reactions. It decreases from high dGTP bias to the lowest dGTP bias (*i.e.*, dGTP 10 nM, other dNTPs at biologically biased concentrations). Although it is possible, especially in the lowest dGTP bias reaction, that the replication complex dissociated from the template such

that an appreciable amount of DNA template was not replicated to completion, and hence the decease in the pfu observed; this is probably not the case in the equimolar (all dNTPs at 100 μM) and the biologically biased dNTP reactions.

Table 2.1

Reversion Frequency of Opal Codon Measured with HeLa or HCT 116 Extracts

extract	replication condition	mutant/total plaques counted	mutant fraction x 10^5
HeLa	untreated	15/532,600	2.82
	equimolar	21/485,350	4.33
	biologically biased	27/681,700	3.96
HCT 116	untreated	9/2,048,250	0.44
	equimolar	59/2,027,925	2.91
	biologically biased	34/1,457,392	2.33

M13mp2SV/opal7 was replicated using HeLa or HCT 116 extracts as described in materials and methods section. "Untreated" refers to mutations when the phagemid template DNA was not replicated with extracts, but directly electroporated and plated onto the plating *E.coli*. In the equimolar and biologically biased reactions, the template DNA was cut with *Dpn I* to completion after the reactions; therefore, blue plaques that are scored from either equimolar or biased reactions would most likely arise from mispairs in the DNA synthesis during the replication reactions, and not from template DNA.

It is possible that in the reactions with dGTP concentrations as low as 10 nM, replication rates are limited, which in turn would affect the observed mutation

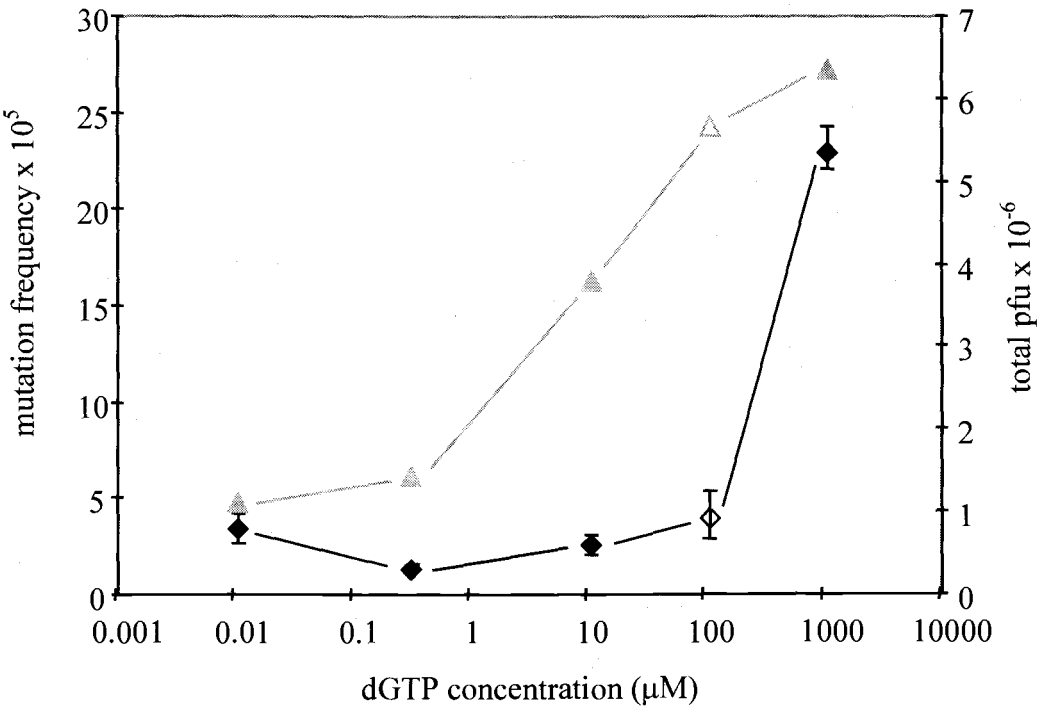
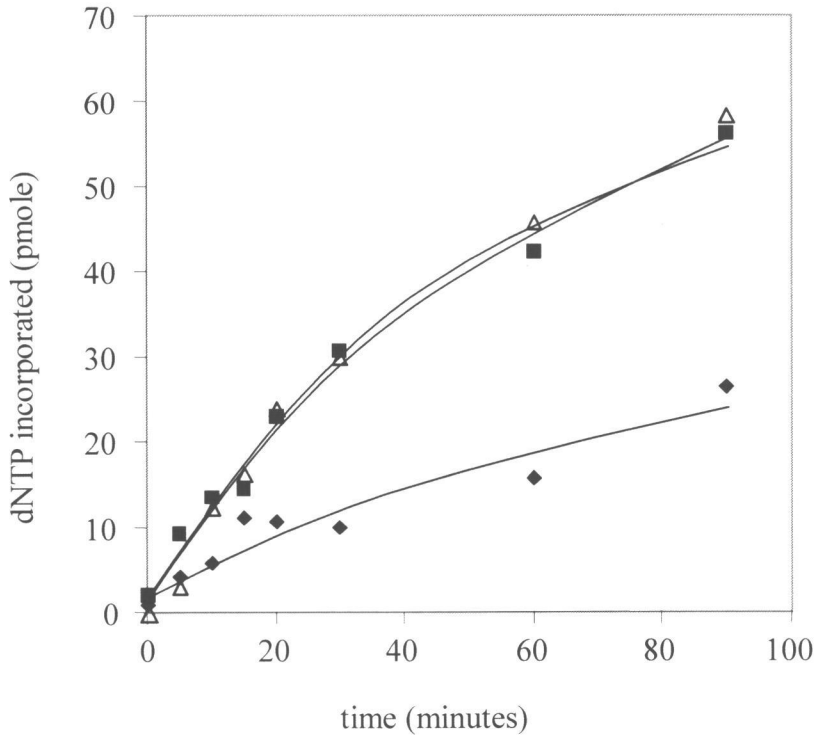


Figure 2.2. Effect of dGTP Bias on Mutation Frequency. M13mp2SV/opal7 was replicated with HCT 116 extracts. All other dNTPs in the reaction mixture for the 100 μM dGTP data point were 100 μM, whereas for all others, the reaction mixtures consist of dATP 60 μM, dTTP 60 μM, dCTP 30 μM and dGTP as indicated. Mutation frequencies were averaged from two different experiments under similar plating conditions. Total pfu (right axis) represents plaque-forming units per 25-μl reaction mixture. Diamonds represent mutation frequency and triangles total pfu values.

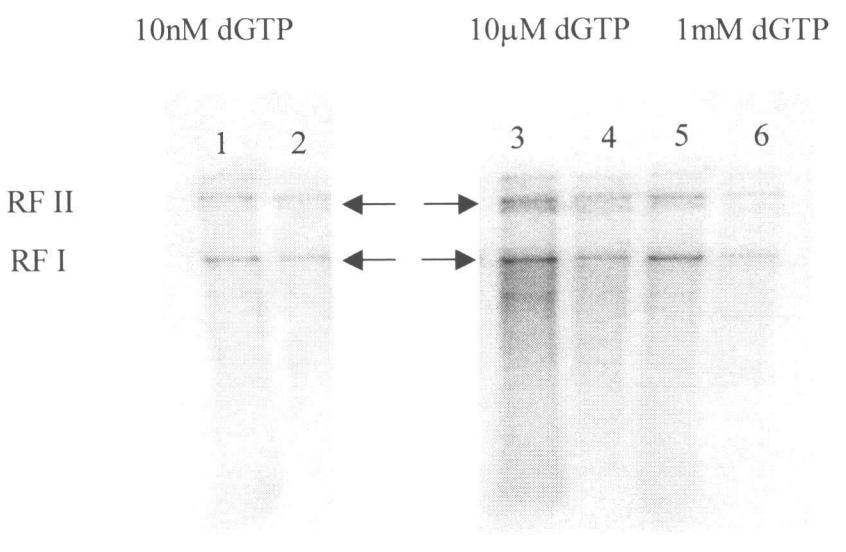
frequency. Measurement of [α-^{32}P]dCTP incorporation showed that the replication rate did decrease appreciably when dGTP concentration was 10 nM (Figure 2.3A). However, there was almost no difference in the replication rates between replication reactions with 10 mM or 1 mM dGTP when all the other dNTPs were held at biologically biased concentrations, suggesting that the estimate of dGTP concentrations in HeLa cells is not rate-limiting in the *in vitro* reaction.

Figure 2.3. Analysis of Replication Reactions with Different dGTP Concentrations. A. Replication rate; diamonds represent 10 nM dGTP; squares, 10 mM dGTP; triangles, 1 mM dGTP. **B**. Autoradiogram of the replication products. Aliquots of each reaction mixture after 90 minutes of incubation digested with *DpnI* (lanes 1, 3 and 5) or *Mbo I* (lanes 2, 4 and 6) and electrophoresed in 1% 0.5x TBE gel at 100 V for about 1 hour. Positions of RF I and RF II were determined in separate analysis.

A


70
60
50
40
30
20
10
0
dNTP incorporated (pmole)
0
20
40
60
80
100
time (minutes)

B


10nM dGTP
10μM dGTP
1mM dGTP
1
2
3
4
5
6
RF II
RF I

Analysis of replication products with *DpnI* or *MboI* showed in Figure 2.3B confirmed that most of the reaction products are hemimethylated since the replication product are resistant to both restriction endonucleases (*Dpn I* cuts methylated DNA and*Mbo I* cuts unmethylated DNA). This result suggests that replication of the template DNA occurred once during the reaction.

EFFECTS OF dGTP OR dTTP BIAS ON "DIALYSED" HELA CELL EXTRACT

To better compare the data with previous studies, the replication reactions were repeated with HeLa cell extracts under condition of low dGTP biases. The HeLa cell extracts were also treated with Centri-sep spin columns to eliminate almost all of the nucleotides present in the extracts; the extracts were termed "dialyzed". The effect of extremely low dNTP biases on mutation frequency was able to be evaluated using the dialyzed extract. Also instead of biologically biased dNTP concentrations, 100 μM of the other dNTP concentrations were used in the reaction for a more comprehensive study of the experimental conditions. Finally, dGTP biases were compared with dTTP biases, to ensure that the effect of dGTP variations was not a specific effect of dGTP on the replication complex.

The Centri-sep column removed 99% of the dNTP present in the extract with no detectable effect on the replication activity as measured by the [α-^{32}P] dATP incorporation assay. Residual dNTP concentrations in 25 μL extract after Centri-sep procedure were 6 nM for dATP, undetectable (< 1 nM) for dCTP, 0.5 nM for dGTP and 4 nM for dTTP. These values were taken into account in the calculations of dTTP or dGTP present in each reaction.

Similar effect of biased dGTP (*i.e.*, low or high dGTP concentration, other dNTPs at 100 μM as shown in Table 2.2) as biologically biased dGTP reactions (low or high dGTP concentration, other dNTPs at biologically biased concentrations as shown in Figure 2.2) on mutation frequency were observed. Again, in the reactions

where dGTP concentration is varied at 1 or 100 μM, the mutation frequencies varied only slightly. Mutation frequency increased about two-fold when dGTP concentration was 1 mM. When dGTP concentration is very low in the reaction (0.0015 μM), 66,000-fold lower than other dNTP concentrations, a four-fold increase in mutation frequency was observed. However, at very low dNTP concentrations, the replication complex could dissociate repeatedly from the template; therefore, the mutation frequency observed is could reflect an inaccurate measure of processive DNA replication fidelity.

The mutation frequencies from replication reactions with dTTP bias (Table 2.2) were very similar to the result for dGTP biases except probably at the lowest concentrations. However, dTTP concentration lower than 4 nM (which is the

Table 2.2.

Effect of dGTP or dTTP Concentration Bias on Mutation Frequency in Replication with "Dialysed" Extracts

dGTP/dTTP concentration (μM)	mutant frequency x 10^5	
	dGTP bias	dTTP bias
1000	12	10.5
100	6	6
1	5	3.7
0.005	--	5.5
0.0015	22	--

The dGTP and dTTP concentrations were varied as shown, while other dNTPs were held constant at 100 μM. dGTP and dTTP concentrations indicated were the sum of added and residual dNTPs present in the "dialysed" HCT 116 extracts.

concentration of dTTP present in "dialysed" HeLa cell extracts) in the reaction could not be assayed. In the reaction with dTTP concentration of 5 nM, 20,000-fold lower than other dNTPs in the reaction, the mutation frequency is increased less than 1.5-fold compared to the mutation frequency of the equimolar dNTP reaction. Perrino and Loeb (1988) did an *in vitro* replication reaction using purified calf thymus DNA polymerase α, ϕX174 *am*3 DNA substrate and low dTTP concentration biases. They found that mutation rate increase was not linear with respect to the concentration biases. However, low dTTP biases in their system increased the mutation frequency significantly. One argument for the different result between the above experiment and this study is in the ability of DNA replication complex of the extracts in the *in vitro* replication reaction system to carry out accurate DNA replication that closely resembles *in vivo* replication reaction (Li and Kelly, 1985) compared to replication reaction carried out only by purified DNA polymerase α. However, another important difference between the two experiments is that in Perrino and Loeb's experiment the concentration of other dNTPs in the reaction was 1 mM, while in the experiment conducted for this study the concentrations of other dNTPs are 100 μM. This difference in the level of other dNTPs in the reaction influences the observed mutation frequency as can be shown subsequently in this study.

SPECTRA OF MUTATIONS AT THE TGA CODON IN REACTIONS WITH BIOLOGICALLY BIASED dNTP AND SISTEMATICALLY HIGH OR LOW dGTP

To understand the effect of dGTP underrepresentation on specific replication errors, individual mutants were sequenced from the sets of replication reactions in which biologically biased dNTP pools with either high or low dGTP concentrations were used (mutants from Figure 2.2). As illustrated in Figure 2.4, nine possible revertants are expected; however, we were only able to score eight different

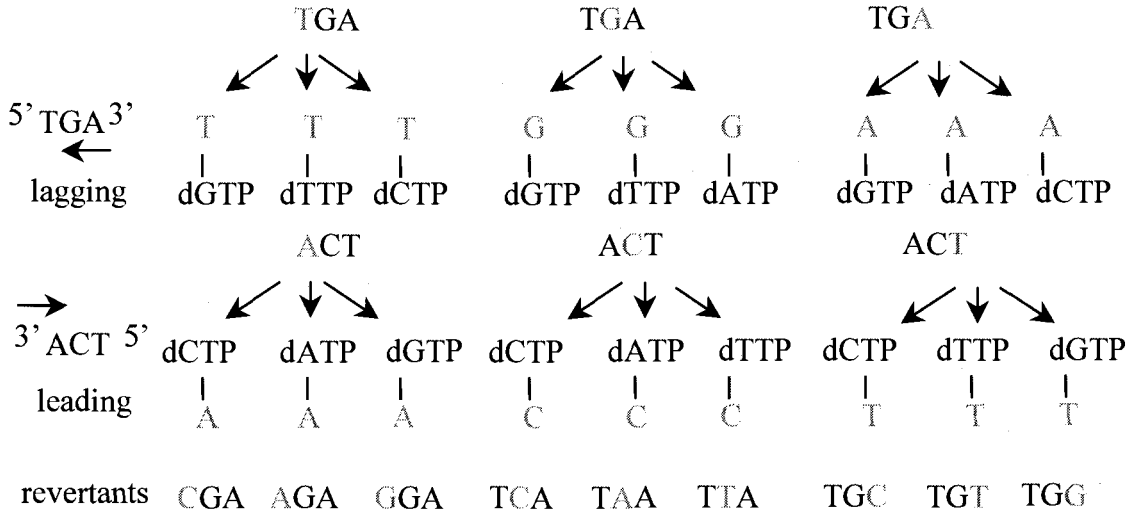
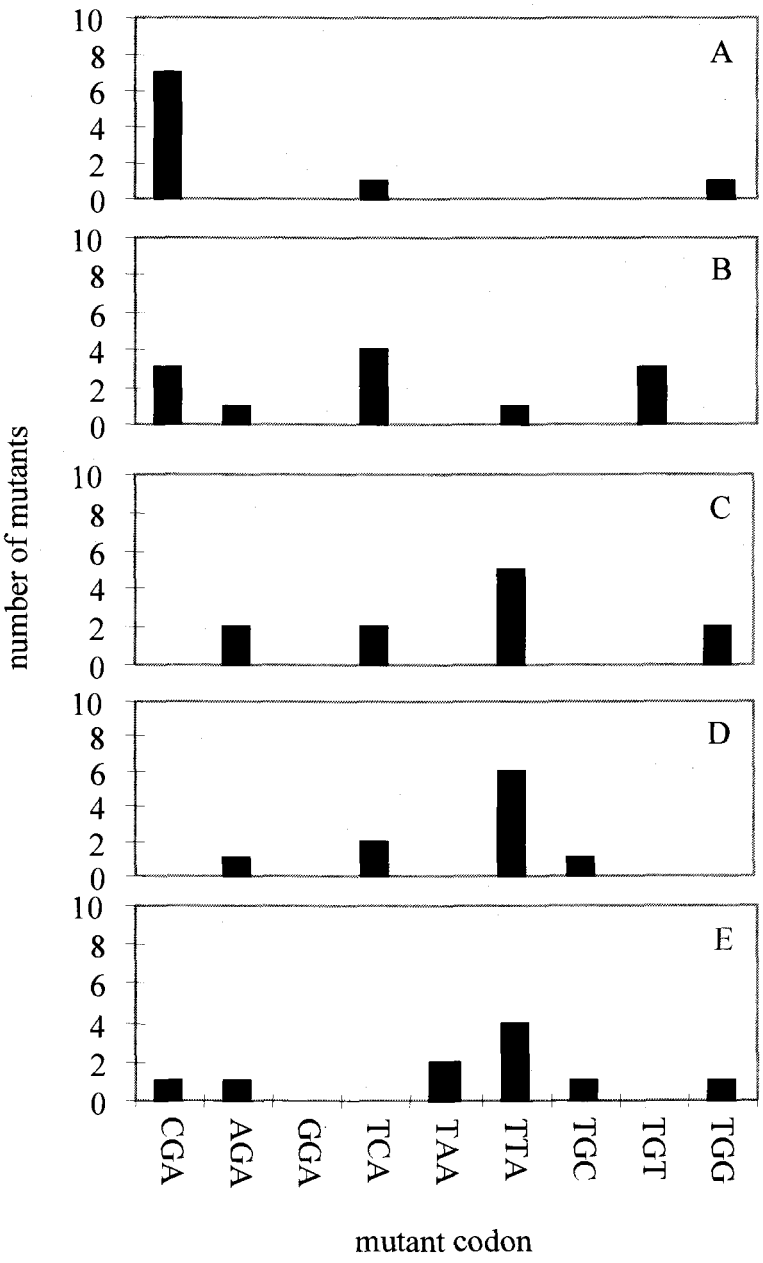


Figure 2.4. Substitution Pathways from Insertion Errors during Replication of an Opal Codon. The resulting revertants from mispairing in lagging strand (first row) or leading strand (second row) are shown directly beneath the mispairs (last row).

revertants. None of the revertants sequenced were GGA, which is the result of either T:dCTP mispair on the lagging or A:dGTP mispair in the leading strand during replication (Figure 2.5). This could suggest that both mispairs were probably not favourably formed or they were corrected very efficiently. A:dGTP mispair leading to GGA revertant is predicted to be one of the most common mispairs in reaction where dGTP is present at high concentration and template A is directly 5' to C during replication (which is represented by "A" on Figure 2.5). Instead of GGA, CGA was the most common revertant obtained in this set of reaction, and GGA was totally absent from the mutation spectrum. It was noted before that A:dGTP mismatch was rarely formed in the replication with human cell extract and unmodified dNTPs (Thomas *et al.*, 1991).

Some other notable results can be concluded from the available sequence data. First, there seem to be different preferences for revertants that are formed in the equimolar as opposed to biologically biased dNTP reactions. On the other hand,

Figure 2.5. Sequence Analysis of Reversions from Opal Codon of the *lacZα* Replicated with Biologically Biased dNTP Concentrations. The sequences represented above are from revertants isolated from replication reactions depicted in Figure 2.2. Panel A, C, D and E represent revertants isolated from replication reaction with biologically biased dATP, dTTP and dCTP concentrations. dGTP concentrations were: 1 mM (high, panel A), 10 μM (biologically biased, panel C), 0.3 μM (low, panel D) and 10 nM (lower, panel E). Panel B represents reactions with equimolar dNTPs (100 μM). Nine mutants were sequenced from high dGTP reaction, 12 from equimolar, 11 from biologically biased and 10 each from low and lower dGTP reaction mixtures.


A
B
C
D
E
10
8
6
4
2
0
number of mutants
CGA
AGA
GGA
TCA
TAA
TTA
TGC
TGT
TGG
mutant codon

biologically bias, "low" (dGTP is 0.3 μM), and to some extent "lowest" (dGTP is 0.01 μM) biologically biased dNTP reactions shared similar revertant representations (Figure 2.5) that are different from the equimolar dNTP reaction. As illustrated in Figure 2.4, each of the revertants can be formed as the result of different mispairing on either leading or lagging strand during replication. In the equimolar dNTP reaction, it is difficult to distinguish whether a specific revertant arose from a mispair in the lagging or leading strand replication. However, in the replication reaction with biologically biased dNTP it is predicted that mispairs on template C and 5' to template C on the leading strand would be more predominant since dGTP is lowest in concentration. TTA, the most prevalent revertant in the biologically biased dNTP reactions, is therefore most probably the result of C:dTTP mispair on the leading strand. TTA is also one of the revertants represented in the equimolar dNTP reaction; however, TCA is the more predominant revertant (Figure 2.5).

Second, from previous experiments using the forward mutation assay of the M13 phagemid and equimolar dNTP concentrations (Roberts and Kunkel, 1988) it was expected that C:dATP mispair would be formed more frequently then either C:dCTP or C:dTTP. C:dATP mispair on the template strand of M13mp2SV/ochre7 would result in an ochre codon; therefore, *E. coli* NR11089, an ochre suppressor, was used to be the α-complementing *E. coli* strain. However, as shown in Figure 2.5, only two out of all the revertants sequenced was TAA. The preference of forming other mispairs than C:dATP at this particular site could be due to the sequence selectivity of the surrounding basepairs, which can affect the mispair formed at a particular site in a DNA sequence.

In conclusion, from the available sequence data it can be stated that there is significant difference ($p = 0.028$, Fischer's exact test) between the mispairs formed opposite template C in replication reactions with equimolar *versus* biased dNTP concentrations; although the amount of sequence data available is small. Predicting possible important mispairs formed *in vivo* using equimolar dNTP replication

reaction, therefore, might not be accurate if in fact biologically biased dNTP pool supplies mammalian DNA replication *in vivo*.

Previously, two effects were predicted of low dGTP concentration on mutation frequency: increase in misinsertion opposite template C and decrease in

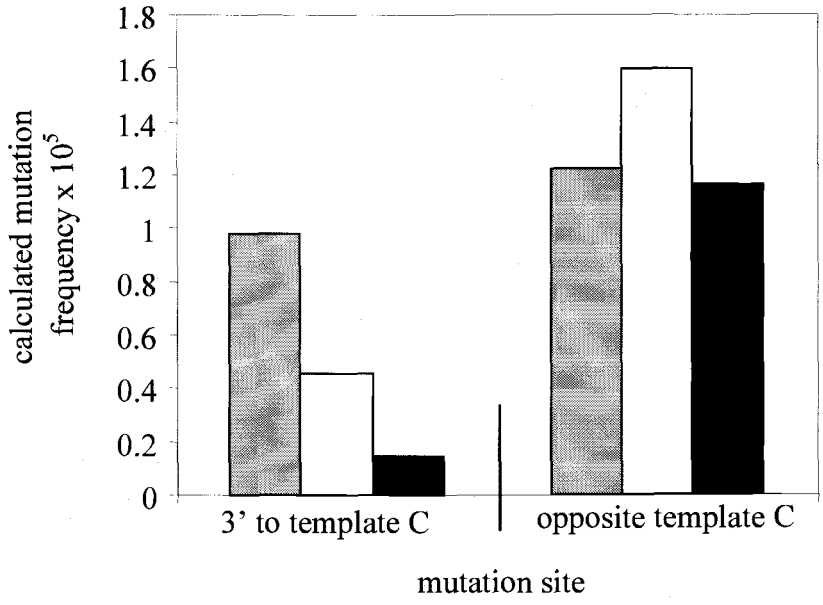


Figure 2.6. Calculated Mutation Frequency within Opal Codon. The calculated mutation frequencies plotted were estimated as proportion of specific mutant class from the total mutants sequenced multiplied by the average mutation frequency for the particular reactions. Data are from experiment represented in Figure 2.5. Grey bars represent equimolar reaction mixture, open bars biologically biased reaction mixture and black bars low dGTP reaction mixture.

mutation frequency 5' to template C. In Figure 2.6, the calculated mutation frequency of revertants at the two different mutation sites of the opal codon: 5' to template C and on template C, was compared. These calculated mutation frequencies were derived from the proportion of sequenced mutations of specific sequences in a particular reaction multiplied by the total mutation frequency of that reaction. The

calculated mutation frequencies of the two positions on the TGA codon can be used to evaluate the effect of underrepresentation of dGTP in each position. Althugh the sequence data were to small to draw conclusive results, it can be stated that the mutation frequency 5' to template C between equimolar, bias, and low bias replication reactions shows a tendency to decrease in that order suggesting the effect of low dGTP in decreasing the mutation fequency.

To distinguish the possibility whether A:dCTP mispair in the leading strand, which would generate CGA revertant for the biologically bias reactions, is either proofread very efficiently when dGTP concentration is low or not preferentially formed in biologically biased dNTP reaction, the DNA template was replicated using HCT 116 extracts, biologically biased dNTP concentration, and either 0.5 mM or 1 mM dAMP to inhibit proofreading (Table 2.3).

Table 2.3

Effect of Inhibited Proofreading on Relication Error Frequency

extract	[dAMP] (mM)	mutant/total plaques counted	mutant fraction x 10^5
----	(untreated)	9/2,048,250	0.15
HCT 116	0	34/1,457,392	2.33
	0.5	30/157,067	19.1
	1	39/143,600	27.2

The reactions were carried out with biologically biased dNTP concentrations in the presence of dAMP as indicated.

As expected, the mutation frequency increases considerably when dAMP was added to the reaction to inhibit proofreading. In the reaction with 0.5 mM dAMP eleven out of eleven revertants sequenced are AGA, which most likely resulted from A:dATP mispair 5' to template C. Therefore, A:dCTP is most probably not preferentially formed.

REPLICATION REACTION WITH TAA CODON

The sequence data thus far indicate that there is a tendency towards a decrease in mutation frequency opposite template C when dGTP is low in concentration. In order to examine the effect of biologically biased dNTP concentration without the added effect of mismatches opposite template C, another DNA construct, M13mp2SV/ochre7, was used which has an ochre codon in place of TGA. Directly 5' to the ochre codon is template C, which is a silent mutation of the original codon (C-TAA). This template was used to hopefully result in a significant decrease in the mutation frequency of the reaction with biologically biased dNTP

Table 2.4

Reversion Frequencies of Ochre Codon in Reactions with Equimolar and Biologically Biased dNTP Concentrations

replication condition	mutant/total plaques counted	mutant fraction x 10^5
untreated	3/629,000	0.15
equimolar	30/1,065,334	2.81
biologically biased	6/267,440	2.24

In the reactions above, the construct M13mp2SV/ochre7 was replicated with HCT 116 extracts.

concentration when compared to equimolar dNTP concentration. As presented in Table 2.4, there does not seem to be any difference between mutation frequencies of the two reactions. However, from nine possible revertants two would produce a stop codon, which would be indistinguishable from the background. As previously discussed, A:dCTP mispair is one of the major mispairs observed for equimolar dNTP reactions at this codon. A:dCTP mispair on either first or second A template of the TAA codon would produce a stop codon. Therefore, the mutation frequency of TAA template replicated with equimolar dNTP concentrations could be an underrepresentation of the actual mutation frequency. In the biologically biased dNTP reaction, instead of A:dCTP, A:dATP mispair constituted the predominant mispair observed. Then it is probably unlikely that TGA or TAG is a major contributor of revertants in the biologically biased reaction since both involved mispairing of A:dCTP. This could be interpreted to mean that the observed mutation frequency of M13mp2SV/ochre7 replicated with biologically biased dNTP concentrations probably closely resembles the actual mutation frequency. Therefore, although indirectly, replication with biologically biased dNTP concentration could result in a lower mutation frequency than replication with equimolar dNTP concentrations. However, more experimentation is still needed.

REPLICATION REACTION WITH "DIPLOID FIBROBLAST" dNTP CONCENTRATION

In general, untransformed mammalian cell lines have lower dNTP concentrations compared to HeLa cell dNTP pools. Table 2.5 shows the calculated the average of each of the dNTP pool concentrations of human diploid fibroblasts measured from IMR90, normal human diploid fibroblast and human fibroblast pool data from other laboratories. By assuming that there is very little difference in the proportion of nuclei to the whole cell volume of HeLa and human diploid fibroblast cells, the concentration of nucleotides present in the human diploid fibroblast cells

Table 2.5

Estimation of Nuclear dNTP Concentration in Human Diploid Fibroblast Cell Lines

cells	dNTP pools (pmole/million cells) dATP	dTTP	dCTP	dGTP	reference
Human fore-skin fibroblast	24	47	12	5	Snyder, 1984
Normal embryonic lung fibroblast	15	31	30	10	Collins and Oates, 1987
IMR 90 diploid fibroblast	30	75	18	8	this study
Normal diploid fibroblast	30	45	20	10	this study
Average	25	50	20	8	
Approximate nuclear concentration, μM	15	15	10	3	

The average values were used to estimate nuclear concentration in μM using data and assumptions of nuclear to whole-cell volumes, and the distribution of dNTPs between nuclear and whole-cell HeLa extracts as reported previously (Leeds *et al.*, 1985).

was calculated (Table 2.5). The effect of replication using the "diploid fibroblast" dNTP pool concentrations on mutation frequency was evaluated. The result shows that mutation frequencies of reactions with diploid fibroblast dNTP concentrations are very much lower than the reaction with 100 μM equimolar dNTP concentration for both constructs involving either a TGA or TAA codon (Table 2.6). The mutation frequencies seen in the reaction with diploid fibroblast dNTP concentrations were

very accurate, resembling the untreated mutation frequency in both cases. Also shown is the rate of replication reaction with diploid fibroblast dNTP concentrations, which was only slightly lower than the rate of the reaction with biologically biased dNTP concentrations calculated from HeLa cell nuclei (Figure 2.7). These data suggest the importance of tight regulation of dNTP pools in controlling DNA replication fidelity without considerably compromising the efficiency of DNA replication in cells.

Table 2.6

Error Frequencies of Replication Reaction with dNTP Concentrations Estimated for Diploid Fibroblast Nuclei

codon	replication condition	mutant/total plaques counted	mutant fraction x 10^5
TGA	untreated	4/202,250	1.97
	equimolar (100 μM)	23/570,000	4.03
	diploid fibroblast	21/3,194,000	0.66
TAA	untreated	3/629,600	0.48
	equimolar (100 μM)	30/1,065,334	2.81
	diploid fibroblast	2/557,400	0.36

Replication reactions were run with HCT 116 extracts.

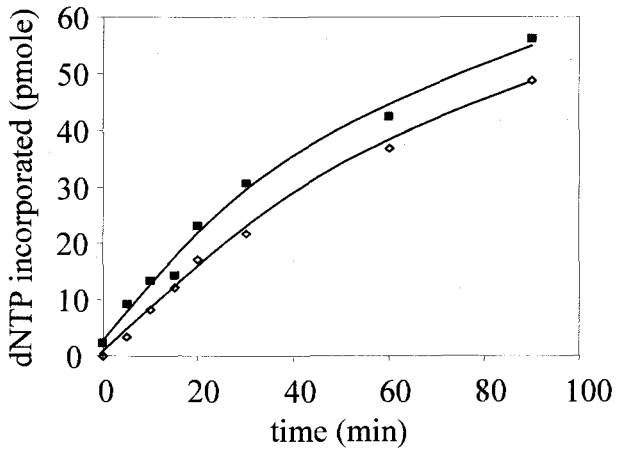


Figure 2.7. Effect of dNTP Concentrations on Replication Rates *in Vitro*. Incorporation of dNTP was monitored by [α-^{32}P]dCTP. Black rectangles represent reaction with HeLa cell dNTP concentrations, open diamonds diploid fibroblast dNTP concentrations.

DISCUSSION

Deoxyribonucleotide concentration during DNA replication is an important determinant of DNA polymerase fidelity (Kunkel, 1992). Asymmetric dNTP concentration can increase the frequency of misincorporation due to the competition of the four substrates to the active site. However, in order for a misincorporation to become a mutation, the mismatch terminus has to be extended and not corrected by the 3'-5' exonuclease proofreading activity. Extending mismatch termini requires a high concentration of the next nucleotide; hence, overall nucleotide levels are also an important determinant of DNA polymerase fidelity (Kunkel, 1988). Another factor contributing to polymerase fidelity is the observation that some mismatches can be extended more easily than others (Creighton *et al.*, 1992), and there is also some evidence of differential proofreading activity on different mismatches *in vivo* (Meuth, 1989). The accumulation of revertants in the replication reaction therefore

depended upon all of the above factors. In the *in vitro* replication reaction, other factors that are actively present in extracts, such as different mismatch repair complexes, could also contribute to the observed mutation frequency.

From the data presented here some notable conclusions can be drawn on the effect of biologically biased dNTP concentrations on DNA replication accuracy *in vitro*. First in this *in vitro* replication system, replication reaction using mismatch repair deficient cell extracts does not seem to have an effect on the observed mutation frequency. Second, replication reactions with biologically biased dNTP pool concentrations calculated from HeLa cell nuclei (Leeds *et al.*, 1985) were not significantly more accurate when compared to the reaction with equimolar (100 μM each) dNTPs. Sequence analysis of revertants shows that in the biological dNTP reaction there can be preferences for forming certain revertants that are different from reactions with equimolar dNTP concentration. Third, an effect that can be inferred from the sequencing is that in the biologically biased dNTP, there is a tendency for a decrease in mutation frequency 5' to template C when dGTP concentration in the reactions were lowered while other dNTPs were kept at biologically biased estimate. Also, next nucleotide concentration of 60 μM (in the case of biologically biased reaction) and 100 μM (in the case of biased dTTP and dGTP reactions) is probably not enough to extend mispairs significantly without correction since lowering the concentration of dGTP several thousand fold, which should increase the probability of mispairs opposite template C, did not tend to increase the mutation frequency opposite template C proportionally.

Biologically biased dNTP concentrations used in most of the experiment in this study are calculated from dNTP pools of HeLa cell nuclei. Untransformed, normal diploid cells in culture, such as human diploid fibroblast cells, have lower whole-cell dNTP pool concentrations than HeLa's. By assuming that the proportions of the nuclei compared to whole cell volume are similar in the two kinds of cell lines in culture the approximate nuclear dNTP concentrations for fibroblast cells were calculated and used for replication reactions. The mutation frequency was not

significantly different from that of the background, which is even lower than seen with the equimolar (100 μM) dNTP concentrations. Extension from a mismatch terminus is probably very unfavorable at the low diploid fibroblast dNTP concentrations thereby decreasing the overall mutation frequency without affecting the replication rate, ensuring high efficiency of replication with high fidelity.

The notion that transformed cell lines with increased overall dNTP concentrations have higher mutation frequency during replication compared to normal dividing human cells with lower overall dNTP concentrations merits further experimentation. Most cells found in human tissues are not rapidly dividing; therefore, except for their mitochondrial dNTP pools, the dNTP pools are negligible. However, it would be interesting to see whether the "abnormally" high dNTP pools (higher than the rapidly dividing normal cell dNTP pools) that these cells seemed to have after they are transformed to cancer cells accounts for the increase in mutation frequency seen with most cancer cells.

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CHAPTER 3

DEOXYGUANOSINE TRIPHOSPHATE LEVEL AND THE RATE OF DNA ACCUMULATION DURING S-PHASE IN HELA CELLS

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SUMMARY

Deoxyguanosine triphosphate (dGTP) concentrations in most mammalian cells represent only 5 –10% of the total whole-cell dNTP pools. This study investigated whether dGTP, being the lowest in concentration in cells, becomes limiting for the rate of DNA synthesis during S-phase in HeLa cells. Aphidicolin was used to synchronize HeLa cells in early S-phase, which enabled the dNTP pools during S-phase to be analyzed in these cells. Although it was found that in particular dATP and dTTP pools were increased by about two-fold in the beginning of S-phase in aphidicolin-treated HeLa cells, the rate of DNA accumulation did not seem to be affected except in the beginning of S-phase. Analysis of dNTP concentrations at hourly intervals during S-phase showed that there was no major change in the ratio of all four dNTP pools through S-phase. Also, addition of aphidicolin during S-phase in HeLa cells seemed to affect dNTP accumulation in early but not in late S-phase, suggesting that dNTPs are probably regulated differently in early than in late S-phase. All four dNTP concentrations were lowest in the middle of S-phase, where the approximate nuclear dGTP concentration was about 2 μM. DNA accumulation is high at the end of S-phase and low in middle and early S-phase. However, there were times in both early and middle S-phase which had a relatively slow DNA accumulation rates. Addition of 8-aminoguanosine and 2'-deoxyguanosine to the cell medium increased the whole-cell dGTP pool approximately two-fold. Hourly examination of DNA accumulation during S-phase with addition of 8-aminoguanosine and 2'-deoxyguanosine 30 minutes before each time point indicates that there could be an increase in DNA accumulation rate in early S-phase when compared to DNA accumulation rate in untreated HeLa cells. However, the possible increase in the DNA accumulation rate did not correspond to the time at which dNTP pool levels were lowest in S-phase. Therefore, more experimentation still needs to be done to address the potential role of dGTP in S-phase replication rate.

INTRODUCTION

The origin of isochores, regions in the mammalian chromosome of compositionally homogeneous GC content (reviewed in Sabeur *et al.*, 1993) has been suggested as the result of slight differences in spontaneous mutation frequency in regions of mammalian chromosomes that are replicated at different times during S-phase (Wolfe *et al.*, 1989). Gu and Li (1994) proposed a model correlating mutation rates with the incidence of GC-rich isochores based on the fluctuation of deoxyribonucleotides (dNTPs) supply during S-phase. Early observations of dNTP levels in mammalian cell lines during S-phase showed an increase in concentrations of all four dNTPs (Adams *et al.*, 1971, Skoog *et al.*, 1973, Leeds *et al.*, 1985). In Chinese Hamster Ovary (CHO) cells, Leeds *et al.* (1985) showed that there was a very significant increase in whole cell and a smaller but still significant imbalanced increase in nuclear dCTP concentration during S-phase compared to the other three dNTPs. However, only a few time points were taken during S-phase, and the increase in dCTP pool was explained as the result of the use of a subline of the CHO cell line lacking dCMP deaminase. Decreased conversion of dCMP to dUMP in these cells causes dCTP to accumulate. A closer examination of dNTP levels during S-phase in cells with dNTP levels more representative of the levels in the majority of mammalian cell lines studied is needed to substantiate the previous observation.

Another question in relation to dNTP levels in S-phase of mammalian cells is whether the concentrations of dNTPs present for DNA polymerases during S-phase affect the rate of DNA replication. The level of dNTP measured in mammalian cells was calculated to be enough only for a few minutes of DNA synthesis (Reichard, 1988). Moreover, dGTP in most mammalian cells is almost always under-represented, contributing only 5 - 10% of the total dNTP pool levels in cells (reviewed by Mathews and Ji, 1992). During S-phase does the level of dGTP concentration drop significantly to affect the rates of DNA synthesis? Previous study on DNA replication rate of HeLa cells during S-phase showed that replication rates

are different in early, middle or late S-phase (Collins, 1978, and Collins *et al.*, 1980). Studies involving DNA fiber radiography to reveal the rate of replication fork extension also suggested that elongation of new DNA is faster towards the end of S-phase (Painter and Schaeffer, 1971, Housman and Huberman, 1975). Therefore, there are data supporting changes of DNA replication rates during S-phase; however, no direct indication exists to show that the level of dGTP in cells during S-phase can modulate DNA replication rates.

In this report the effect of different synchronization drugs on dNTP pool levels in cells was examined, the effect of aphidicolin in several cell lines available compared, and the dNTP pool levels and the average DNA accumulation rate in aphidicolin-synchronized HeLa cells were measured. The whole-cell dGTP pool was increased by addition of 8-aminoguanosine and 2'-deoxyguanosine to the cell medium, and the effect of increased dGTP pool on DNA accumulation rate during S-phase was examined.

MATERIALS AND METHODS

CELL LINES AND REAGENTS

HeLa and HeLa S3 cell lines were obtained from American Type Culture Collection (ATCC). The cells were maintained in DMEM/F-12 medium, high glucose (Gibco BRL) supplemented with 10% fetal bovine serum (FBS). Normal diploid human foreskin fibroblast untransformed cell line was obtained from Dr. David Barnes. The fibroblast cells were maintained in DMEM low glucose medium (Gibco BRL) supplemented in 10% FBS. IMR 90 diploid lung fibroblast cell line was obtained from ATCC, and maintained in E-MEM medium supplemented with 10% FBS, 1.5 g/L sodium bicarbonate and 1 mM sodium pyruvate. Chinese Hamster

Embryo Fibroblast (CHEF/18) cell line was obtained from Dr. Prem Reddy, and was grown in DMEM high glucose medium containing 5% FBS and 4 mM L-glutamine. Stock solution of aphidicolin was 10 mM in dimethyl sulfoxide (DMSO) and stored at 4°C. Mimosine (Sigma) was made up as 100 mM stock solution in phosphate buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L double-distilled H_2O (ddH_2O), prepared as 10x solution, autoclaved). The stock solution for ciclopirox-olamine (Sigma) was made as 5 mM in PBS. Roscovitine (Calbiochem) was made as 10 mM in DMSO and stored at 4°C. 8-aminoguanosine and 2'-deoxyguanosine were made as 10 mM and 100 mM in DMSO respectively, and guanine was made as 100 mM in DMSO.

CELL SYNCHRONIZATION

Synchronization with aphidicolin was done as described by Matherly *et al.* (1989) with some optimization. The doubling time of HeLa cells in culture was about 24 hours. The time the cells spent in S-phase was typically one-third of the duration of the cell cycle; therefore, for HeLa cells, S-phase should be about 8-10 hours. G1 phase would be the longest of the cells' phases. To synchronize the cell population at G1/S border or early S-phase, aphidicolin at a final concentration of 3 μM was added to log-phase growing cells (immediately or 24 hours after the cells were trypsinized and regrown in a new plate), for the indicated times for the first block. The medium was then aspirated, the cells washed with prewarmed PBS, and new medium was added to the cell, which were then incubated for another 15 hours (termed "release"). Aphidicolin was added to the medium, and the cells incubated for another indicated period of time. Upon washing with prewarmed PBS and adding new medium to the cells, the cells immediately begin synthesizing DNA, as confirmed by flow cytometry analysis.

Additions of various drugs to the HeLa cells were done immediately after the cells were reseeded onto new plates. The drugs were added at concentrations noted on previously published data on cell synchronization (mimosine, final concentration of 100 μM; roscovitine, 10 μM and ciclopirox-olamine, 15 μM) and for either 6, 8 or 10 hours. For effect of aphidicolin on various cell lines, the drug was added to final concentration of 3 μM to the cells' medium for one or two hours before the dNTPs were extracted and measured.

FLOW CYTOMETRY ANALYSIS

At the indicated time points, cells were harvested by trypsinization, moved to a 50-mL conical centrifuge tube and washed twice with ice-cold PBS. Centrifugations were done at 1,500 rpm, for 2 minutes, at 4°C in a Beckman swinging bucket table-top centrifuge. After the second wash, the pellet of cells was resuspended in PBS left in the 50-mL conical tube after aspiration. 2 mL of ice-cold 70% ethanol-H_2O (v/v) was then added dropwise while the cells were agitated with a vortex mixer. The cell suspension is fixed and can be left in the refrigerator for up to a week.

Immediately before flow cytometry analysis, the ethanol-fixed cells were centrifuged at 1,500 rpm for 2 minutes using the same rotor, aspirated and resuspended in 500 μL of 1 mg/mL RNaseA in phosphate buffer solution (50 mg RNase A, 279 mg of $Na_2HPO_4 \cdot 7H_2O$, 972 mg of $NaH_2PO_4 \cdot H_2O$ in 50 mL of ddH2O, filter sterilized). The cells were incubated at 37°C for 10 minutes to degrade the RNA present in the cells. The cells were stained with propidium iodide (PI), which intercalates in both DNA and RNA; therefore, digesting the RNA present in the sample to completion was essential in obtaining high quality flow cytometry profile of the cells' DNA content. After RNaseA digestion, 200 μl of 300 μg/ml PI solution in PBS was added to each cell suspension, and the cells were incubated for 20

minutes at room temperature in the dark. Thereafter the experiment was done in the dark. The cell suspension was filtered through a 40-micron mesh immediately before flow cytometry analysis. The flow cytometry machine (Coulter EPICS XL, Beckman Coulter, Inc., Miami, FL) is courtesy of Environmental Health Sciences Cell and Tissue Analysis Core facility, Oregon State University.

It is a good idea to have an untreated log-phase growing sample of the cells to be able to set a correct gating for cells to be analyzed. Sometimes changes in the cells or materials (age of PI solution for example) can change the fluorescent intensity gating; therefore, a normal growing cell culture treated at the same time in each experiment can provide controls for flow cytometry gating parameters.

dNTP POOL AND DNA ACCUMULATION RATE MEASUREMENTS

dNTP Pool Extraction

dNTP pool extraction is done by a quick extraction method. At the indicated time points, the medium from the cells growing in plates was quickly aspirated. The cells were washed twice, as quickly as possible, with 2 mL of ice-cold Tris-buffered saline (TBS), after which each plate was left standing on its side for 30 seconds to let all the TBS drain; next the plates were aspirated. Immediately, 3 mL of ice-cold 60% (v/v) methanol, containing 1% (v/v) toluene was added to each plate, the plate was rocked a few times and put in –20°C for 1 hour with occasional rocking. After, the suspension from each plate was distributed into 3 1.5-mL Eppendorf tubes. Another 1 mL of 60% methanol, 1% toluene solution was used to wash each plate, and it was also distributed among the Eppendorf tubes. Then the methanol extracts were centrifuged at 14,000 rpm in a fixed-angle Eppendorf microcentrifuge for 30 minutes at 4°C. The supernatant fraction was put into a clean tube and taken into dryness with

a Speed-Vac (Savant). The residue in each tube from one plate was pooled into one tube by solubilizing them in a total volume of 150 μL of ice-cold 5% trichloroacetic acid (TCA). Immediately following the 5% TCA treatment, 1.5 volumes of 0.5 M tri-N-octylamine in Freon solution was added to each tube on ice, and each tube was vortexed at high speed for 90 seconds. They were then immediately centrifuged at 10,000 rpm in the same rotor at 4°C for 10 minutes. The aqueous phase was put into a clean tube, and brought to dryness in the Speed-Vac. The residue from two or three plates per time point was resuspended in 100 μL of ddH2O or dNTP pool assay buffer (45 mM Tris-HCl, pH 8.3), and it was ready for dNTP pool assay.

dNTP pool measurement is as described before in Chapter 2.

DNA Accumulation Rate

The rate of DNA accumulation at each time point during S-phase was approximated by measuring the change in the peak of DNA intensity of the cells' DNA content profile obtained from flow cytometry from one time point to the next, averaged over the difference in time between the two time points. The fluorescence intensity is linearly correlated to the DNA content of the cells. The percent synchrony at each time point was not accounted; therefore, the DNA accumulation rate presented served as an approximation of the average DNA accumulation rate over the indicated times in S-phase.

Treatment with guanine (final concentration 0.1 mM) or 2'-deoxyguanosine plus 8-aminoguanosine (each to final concentration of 0.1 mM) was done every 30 minutes before the next time points to fix the cells for flow cytometry.

RESULTS

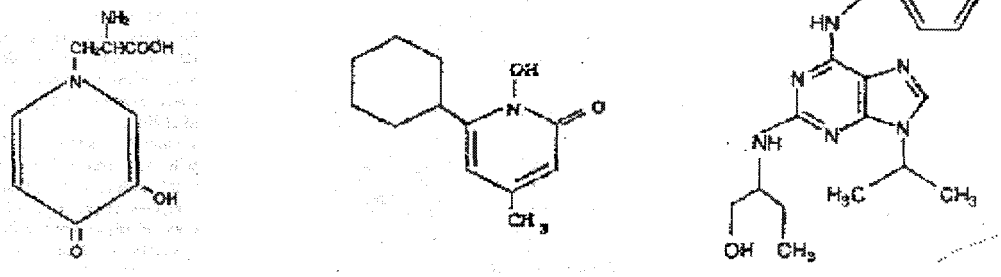
EFFECT OF SYNCHRONIZING AGENTS ON CELL dNTP POOLS

To analyze dNTP pool modulations during S-phase, the cells needed to be synchronized at the beginning of S-phase or at the G1/S phase border without affecting their dNTP pools. Most of the common G1/S or early S-phase synchronizing agents that were tried changed the dNTP pools of cells when compared to the untreated controls, although it seemed that the effects of the drugs vary in different cell lines used. However, the changes that were observed could not be concluded to be not "normal" changes of dNTP pools in unperturbed cells at G1/S or S-phase since the dNTP pool levels of cells at the specific phases of the cell cycle could not be evaluated without the addition of the synchronization drug. Some drugs such as hydroxyurea are known to act directly to decrease the dNTP pools to keep the cells in G1 phase; therefore, the use of this drug for the synchronization procedure was avoided. Effects of other drugs that can be used for G1/S border synchronization, namely, aphidicolin, mimosine and ciclopirox olamine (see Figure 3.1 for structures), on cells' dNTP pool levels were evaluated. The effect of another drug, roscovitine [2-(1-ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine] (Figure 3.1), which acts very specifically to inhibit cyclin-dependent kinase cdk2 (Meijer *et al.*, 1997), was also evaluated. This drug arrests cells at G2/M, and cells accumulate at G1/S border.

Aphidicolin is a drug that binds to DNA polymerases α, δ and ε (reviewed in Malkas, 1998), and can be used to synchronize cells in early S-phase (Pedrali-Noy *et al.*, 1980). As shown in Table 3.1, addition of 3 μM aphidicolin to log-phase growing HeLa cells for one hour increased the steady-state dATP, dTTP and dGTP pools in these cells. Earlier reports on administration of aphidicolin in 3T3 and V79 cells showed similar increase in at least the dATP pool levels in these cells (Bianchi

et al., 1992). Addition of aphidicolin to log-phase growing CHEF 18 and IMR 90 cells showed an increase in dATP, dTTP and dCTP levels with a similar increase in dGTP level for CHEF 18 cells. On the other hand, administration of the drug to log-phase growing human diploid fibroblast cells for one hour did not seem to change the steady state dATP, dTTP or dCTP levels, but dGTP was lowered in these cells.

Since there are changes in the dNTP pool levels when most cell lines were treated with aphidicolin, the effects of other drugs available to synchronize cells at G1/S border were evaluated. Roscovitine is not normally used for synchronization; however, since it has a very specific inhibition for cdk2, the drug was administered to freshly plated HeLa cells for either 6, 8 or 10 hours (Figure 3.2). As shown, after 10 hours exposure to the drug, all four dNTP pool levels were reduced considerably. Furthermore, after release even from 6 hours of roscovitine block, the cells exhibited a significant lag period before entering S-phase, and the effect of the drugs was not completely reversible.



Mimosine Ciclopirox olamine Roscovitine

Figure 3.1. Structures of the Different Synchronization Drugs.

Mimosine can be used to synchronize cells in G1/S border. Some reports have suggested that mimosine arrests cells in late G1 (Lalande, 1990); others have

reported that it arrests cells at origin of replication (Mosca *et al.*, 1992) or after the onset of S-phase (Hughes and Cook, 1996). It seems that mimosine acts to synchronize cells in a complex way perhaps involving more than one mechanism, one of which is through inhibition of ribonucleotide reductase (Gilbert *et al.*, 1995). In HeLa cells addition of mimosine to freshly seeded cells for 6, 8 or 10 hours decreased all dNTP pool levels to different extents compared to the untreated cells (Figure 3.2). Previous observation in Chinese Hamster Ovary C400 (CHO) cells treated for 6 hours with mimosine also showed an effect on the cells' dNTP pools. In

Table 3.1

The Effect of Aphidicolin on Unsynchronized Cells

cell lines	% of untreated cells			
	dATP	dTTP	dGTP	dCTP
CHEF 18	154	148	123	148
Diploid fibroblast	96	98	77	108
IMR 90	168	136	83	126
HeLa	132	133	131	99

Aphidicolin (3 μM) was added for one hour to the medium of log-phase growing HeLa, CHEF 18, IMR 90 and human diploid fibroblast cell lines. dNTP pools were extracted and assayed as in Materials and Methods section.

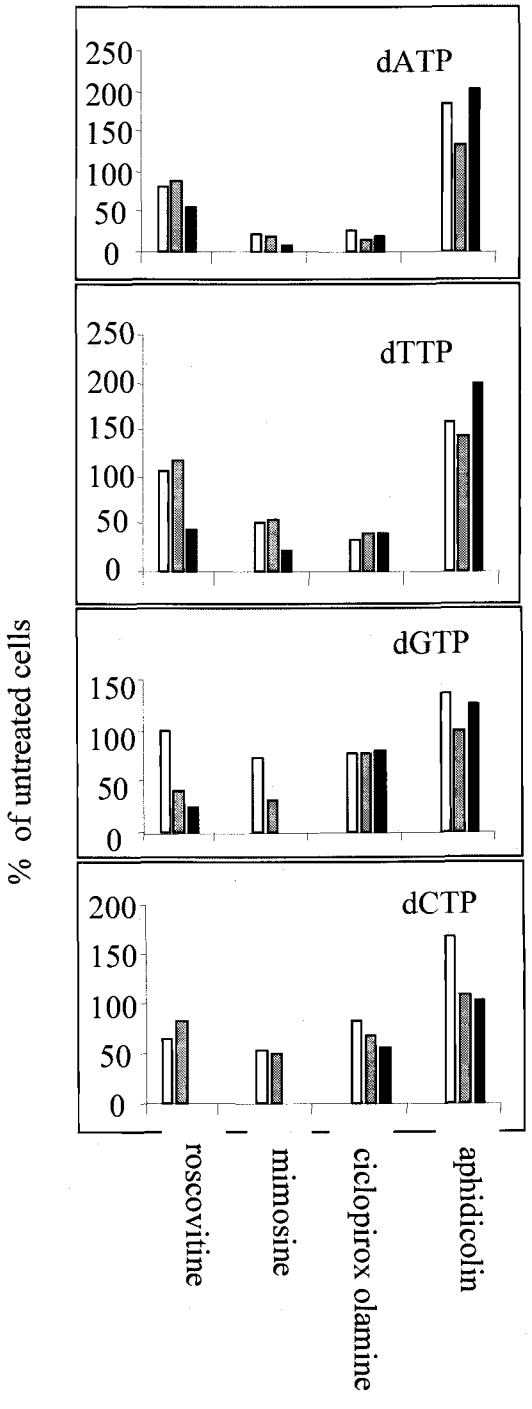
these cells, some decreases were observed in dGTP and dATP pools, the dCTP in these cells was not changed, and the dTTP pools increased considerably (Gilbert *et al.*, 1995). These effects on CHO cells were not completely in agreement with the results that was observed in this report; however, the cells in this experiment were treated with 0.1 mM instead of 0.4 mM mimosine as in the CHO study. The effects

on CHO cells are very similar to the effect of hydroxyurea on cells' dNTP pool. Hydroxyurea is known as an iron chelator, affecting the dNTP pools by inhibiting the ribonucleotide reductase enzyme. Mimosine is thought also to affect ribonucleotide reductase by chelating iron as does hydroxyurea (Kulp and Vuilliet, 1996). These results demonstrate that mimosine disturbs the dNTP pool levels of the cells during synchronization.

Another synchronizing agent, ciclopirox olamine was shown to reversibly inhibit HL-60 promyeloid leukemia cells at G1/S phase boundary (Hoffman *et al.*, 1991). It was also shown as one of the synchronization drugs that did not disrupt cell cycle integration, defined as cyclin A and B accumulations, when S-phase progression was blocked (Urbani *et al.*, 1995). In fact, Urbani *et al.* (1995) showed that prolonged exposure (24-36 hours) to aphidicolin and mimosine resulted in disruption of nucleic and cytoplasmic cell cycle progression in HeLa S3 cells. However, addition of ciclopirox olamine to freshly plated HeLa cells for 6, 8 and 10 hours showed greater than 50% decrease in dATP and dTTP levels in the cells compared to the untreated cells (Figure 3.2). dGTP and dCTP levels in the treated cells, especially for 6-hour treatment, was not as greatly decreased.

Addition of aphidicolin to freshly plated HeLa cells for 8 and 10 hours did not substantially change the dGTP and dCTP pools, although dATP and dTTP pools increased as seen previously with the one-hour treatment of aphidicolin to HeLa cells. Also, there was more accumulation, 1.5- up to 2-fold for dATP and dTTP, when the cells were exposed to the drug for 10 hours (Figure 3.2). However, the cells proceeded through S-phase with very good synchrony after release from a block as short as 8 to 10 hours (Figure 3.3). The increase in the pools also did not change greatly the ratio of one pool to another, dTTP>dATP>dCTP>dGTP; therefore, we decided to analyze HeLa cells dNTP pool dynamics during S-phase using a short aphidicolin block to synchronize the cells in early S-phase.

Figure 3.2. The Effect of Various Synhronization Drugs on HeLa Cells' dNTP Pools. The different synchronization drugs were added to freshly seeded HeLa cells' medium for either 6, 8 or 10 hours, and dNTP pools from the cells were extracted and assayed as described in the Materials and Methods section. dNTP pools are expressed as percent of untreated cells. Open bars represent treatment for 6 hours, grey bars treatment for 8 hours, and black bars treatment for 10 hours. The absence of black bars on several 10-hour treatments represents undetectable levels of either dCTP or dGTP.


dATP
250
200
150
100
50
0
dTTP
250
200
150
100
50
0
dGTP
150
100
50
0
dCTP
200
150
100
50
0
% of untreated cells
roscovitine
mimosine
ciclopirox olamine
aphidicolin

HELA CELL SYNCHRONIZATION WITH APHIDICOLIN

HeLa cells synchronization with aphidicolin resulted in a very good degree of synchrony throughout S-phase as shown in the DNA content profile of the cells collected at the indicated time points throughout the cycle (Figure 3.3). Two aphidicolin blocks were used to achieve high degree of synchrony as reported by Matherly *et al.* (1989). After first addition of aphidicolin to the cell culture medium for as long as 20 hours the cells were washed with PBS, and new medium was added to the cells, which were then incubated for another 15 hours. The second aphidicolin treatment was done for varying times as indicated (Figure 3.4). The dNTP pool levels in HeLa cells during the course of the treatment are shown in Figure 3.4. The figure shows the pool levels at different times during release from the first block, and the accumulation of mainly dATP in the prolonged second aphidicolin block. After 20 hours of first block with aphidicolin, the dNTP pool concentrations in the synchronized cells were much higher than the concentrations in the unsynchronized cells, and dATP level was higher than dTTP, which was normally the largest pool in HeLa cells. High concentration of dATP would inhibit the activity of ribonucleotide reductase in human cells. Therefore, it is reasonable that after such a long halt of DNA synthesis after crossing the G1/S border the cells would eventually suffer inhibited dNTP production. High concentration of dATP has also been shown to aid in the caspase 3 maturation in the caspase cascade which leads to one of the cells' apoptotic pathway (Oliver *et al.*, 1996, Leoni *et al.*, 1998). Prolonged exposure to aphidicolin (36 hours) has been shown to increase the degraded DNA peak characteristic of cells that underwent apoptosis in the DNA content profile by flow cytometry (Urbani *et al.*, 1995).

One aphidicolin block immediately after the cells were seeded onto a new plate gave as good synchrony as the double aphidicolin block that was used earlier. Closer comparison of dNTP pools taken at a few time points during S-phase from cells that were treated with aphidicolin for 13 hours *versus* 20 hours showed that the

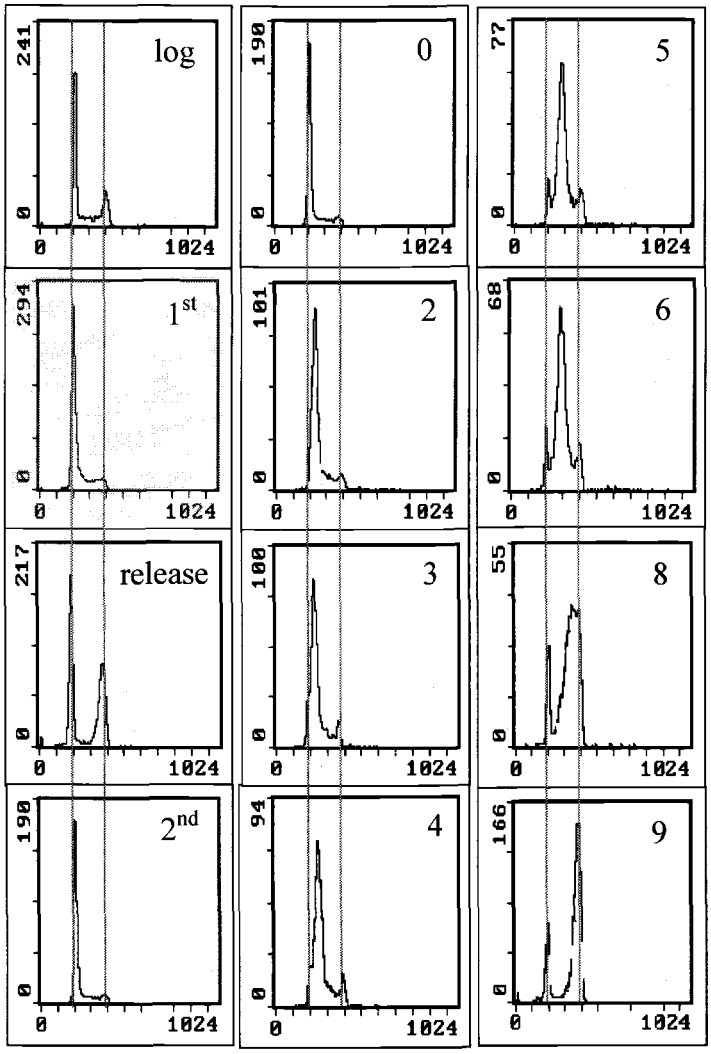
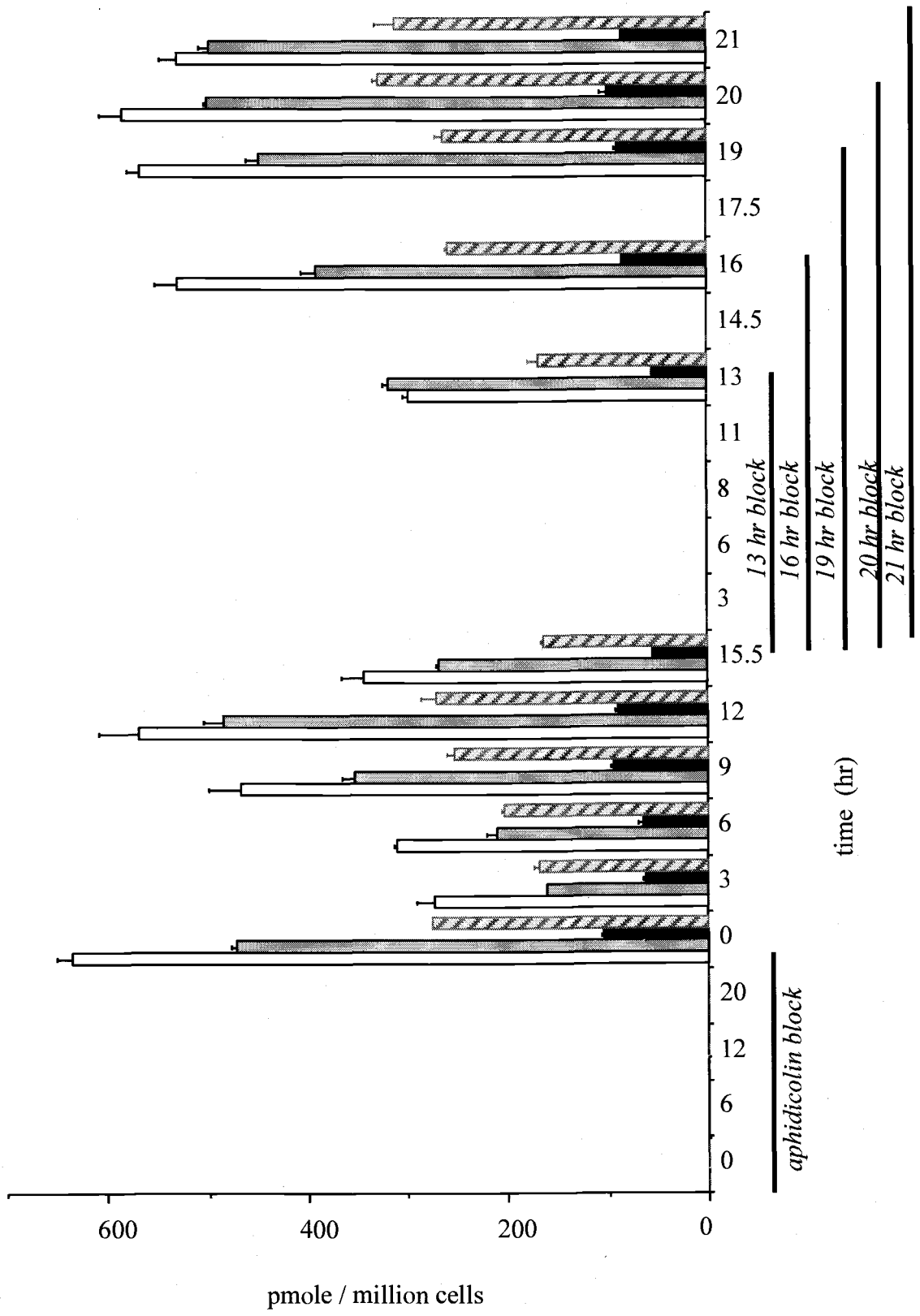


Figure 3.3. Cell Cycle Analysis of Aphidicolin-Treated HeLa Cells. HeLa cells were synchronized with two aphidicolin blocks, and after release from the second block most of the cells proceeded through S-phase with high degree of synchrony. Y-axis indicates number of cells and x-axis fluorescent intensities that are proportional to DNA contents of cells. First column of profiles shows DNA contents of cells during aphidicolin synchronization, and next two columns of profiles shows the cells' DNA contents during S-phase at the indicated times in hours (inserts).

Figure 3.4. dNTP Pool Levels in HeLa Cells during the Course of Aphidicolin Synchronization. Open bars represent dATP, grey dTTP, black dGTP and striped bars dCTP. Error bars represent averages of duplicate experiments.


21
20
19
17.5
16
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13
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8
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3
15.5
12
9
6
3
0
20
12
6
0
13 hr block
16 hr block
19 hr block
20 hr block
21 hr block
aphidicolin block
time (hr)
600
400
200
0
pmole / million cells

difference between the two sets of pools was mainly at the first time point, the zero time point immediately after release from the block (Figure 3.5). Jackson (1995) noted that in 'agarose-trapped' nuclei of HeLa cells, S-phase progression resumed with the expected early, middle and late characteristic replication patterns after a short adjustment period after the cells were released from up to 16 hours of aphidicolin block.

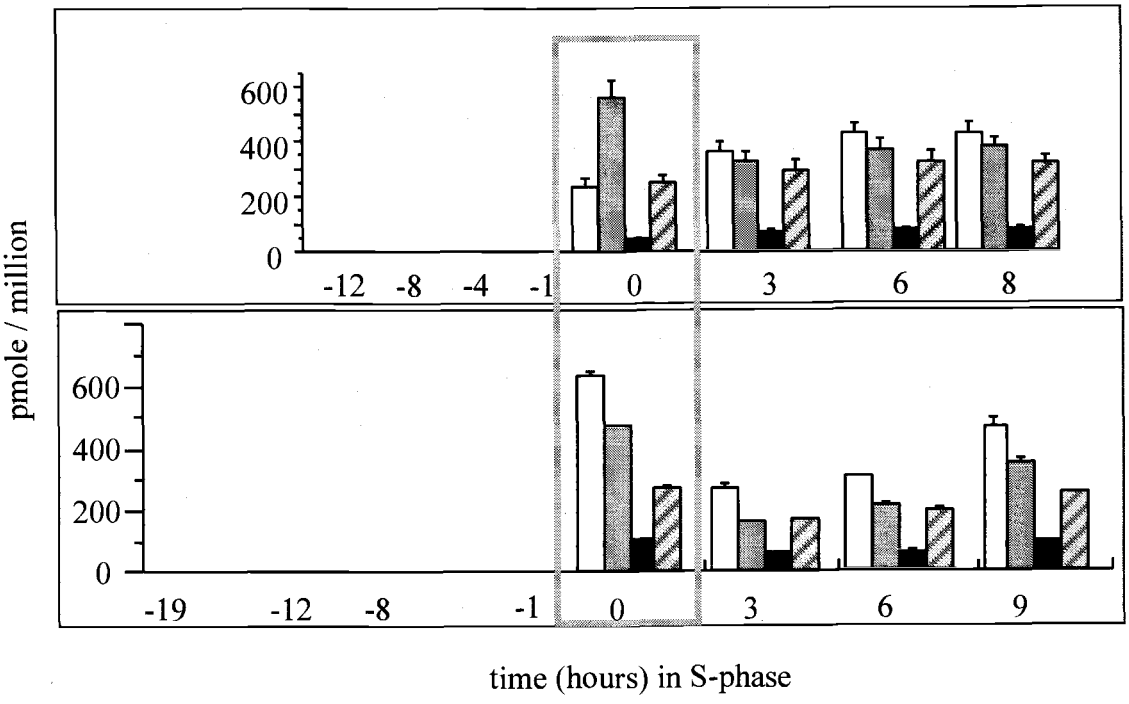


Figure 3.5. Comparison of dNTP Levels in S-phase of HeLa Cells Synchronized with Either 13 Hours or 20 Hours of Aphidicolin Block. dNTP pools were extracted at the indicated time points and assayed. The top figure is dNTP levels in S-phase after 13 hours aphidicolin block and the bottom figure is after 20 hours aphidicolin block. Grey lined box denotes the main difference of the dNTP pool levels between the two sets of experiments. Open bars represent dATP, grey bars dTTP, black bars dGTP and striped bars dCTP. Error bars represent averages of the values obtained from duplicate experiments.

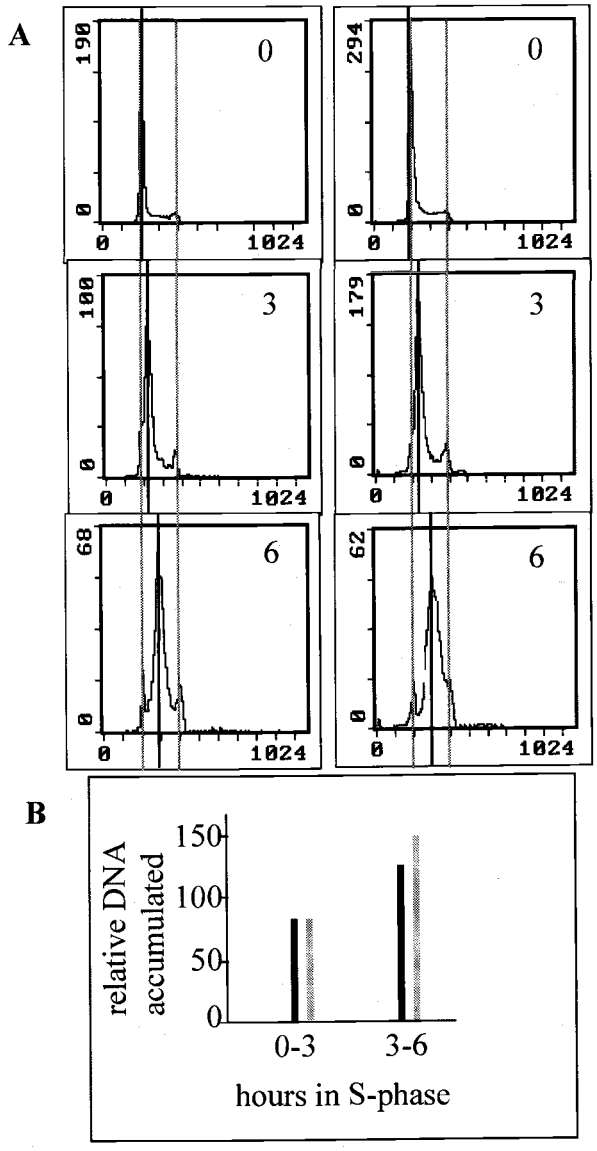


Figure 3.6. Comparison of DNA Accumulation Rates in HeLa Cells Treated with 13 Hours *versus* 20 Hours Aphidicolin Block. A. DNA content profiles of HeLa cells in S-phase after 13 hours aphidicolin block (left flow cytometry profiles) and after 20 hours aphidicolin block (right profiles) were deduced to give DNA accumulation rates in either 0 to 3 hours or 3 to 6 hours (time points are shown by the inserts) in S-phase (the peak of DNA intensity as shown by black lines for hours 3 to 0 or 6 to 3 in S-phase were subtracted to give the relative DNA accumulation rates). X-axis represents cell numbers and y-axis the fluorescent intensity. **B.** Schematic diagram of the resulting relative accumulation rates. Black lines are for 13 hours' synchronization, and grey for 20 hours' synchronization.

When the DNA content of the cells in S-phase from the two different sets of cells were analyzed, there was no considerable difference between DNA accumulation rates in those cells (Figure 3.6), although there was some difference between the DNA accumulated in the first three hours *versus* the second three hours of S-phase in both sets of cells. It was noted that dNTP pool levels of HeLa cells in S-phase after either 13 hours or 20 hours' block were appreciably higher than the steady-state dNTP pools of HeLa cells. However, previous studies have also shown that whole-cell dNTP pools during S-phase increased by least two-fold compared to the pools at other time in the cell cycle (Snyder, 1984, Nordenskjold *et al.*, 1970, Leeds *et al.*, 1985). Therefore, I estimated that data on HeLa cells' dNTP pools synchronized in early S-phase using a 12-hour aphidicolin block would approximate the dNTP pool levels in the S-phase of an untreated cell.

HELA CELL dNTP POOLS DURING S-PHASE

Previous observation of dNTP concentrations during S-phase that was done in our laboratory by Leeds *et al.* (1985) showed an imbalanced increase of dCTP during S-phase in the CHO cells that were used. The dNTP pool levels in S-phase need to be evaluated more closely (at more time points in S-phase), using a cell line which has a more typical ratio of steady state dNTP pools. For this study, the dNTP pool levels in S-phase of HeLa cells synchronized with aphidicolin for 12 hours were analyzed.

After aphidicolin synchronization when aphidicolin was added back to the medium after release from aphidicolin block, the cells ceased DNA synthesis. DNA content profile showed that there is no accumulation of DNA between the drug addition and the next time point, one hour later. When DNA synthesis was blocked early in S-phase, dATP and dTTP pools increased about 150%, similar to the increase observed for these two pools during aphidicolin synchronization (Figure

3.7). dCTP pool was increased by a smaller amount. However, aphidicolin block early in S-phase restored the otherwise depleted dGTP pools, suggesting that dGTP did not undergo the turnover mechanism when replication is blocked at this point. In contrast to the effect of DNA replication block during early S-phase on the pools, blocking replication at the middle of S-phase did not seem to result in a notable accumulation of dATP, dGTP and dCTP pools in cells. There was a notable accumulation of dTTP in late S-phase; however, the extent of accumulation was not as great as when the cells were blocked in early S-phase. This result could suggest different regulation mechanisms of dNTP production and turnover rates in early compared to middle or late S-phase.

Closer examination of dNTP pool levels in S-phase showed a drop of all dNTP pool concentrations in the middle of S-phase that was not seen when only three or four time points were taken during S-phase (Figure 3.8A). dATP concentration was lower than that of dTTP in the beginning of S-phase but towards middle or late S-phase, dATP concentration was higher than dTTP; dATP became the highest of all four dNTP. dGTP concentrations, although increased at the beginning of S-phase, dropped lower than HeLa cells' steady state dGTP concentration in the middle of S-phase.

Is the drop in dGTP pool significant enough to slow replication in the middle of S-phase? It was previously noted that S-phase DNA replication in HeLa cells consists of slow and fast replication rates, with slow phase in the middle of S-phase (Collins, 1978, Collins *et al.*, 1980), although other reports have also suggested that the rate was slowest in early S-phase and fastest towards the end of S-phase (Painter and Schaeffer, 1971, Housman and Huberman, 1975). When the DNA accumulation rate *versus* time from the DNA content profile was examined, the rate of accumulation did not seem to correspond well to the dNTP pool levels at the indicated time points (Figure 3.8B). The highest increase in DNA accumulation rate seemed to occur at the late S-phase stage, and although lower DNA accumulation

Figure 3.7. Effect of Inhibition of DNA Synthesis on dNTP Pool Levels of HeLa Cells during S-Phase. dNTP pools accumulated when DNA synthesis was inhibited in early S-phase. Only dTTP showed a slight accumulation of pools when DNA synthesis was inhibited in middle or late S-phase. Black bars are dNTP levels during continuous S-phase in HeLa cells, and grey bars are dNTP levels after aphidicolin was added back to medium at the indicated time points (black arrowheads).

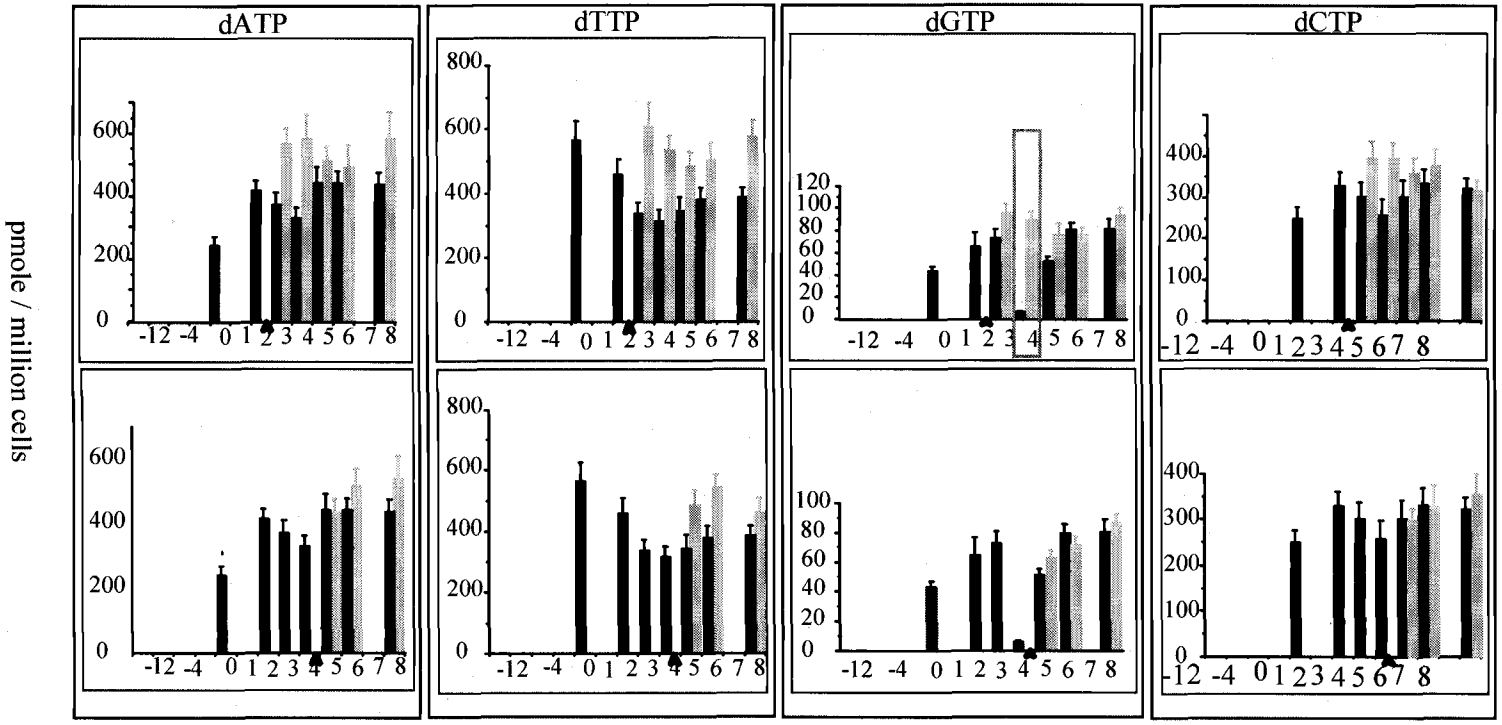
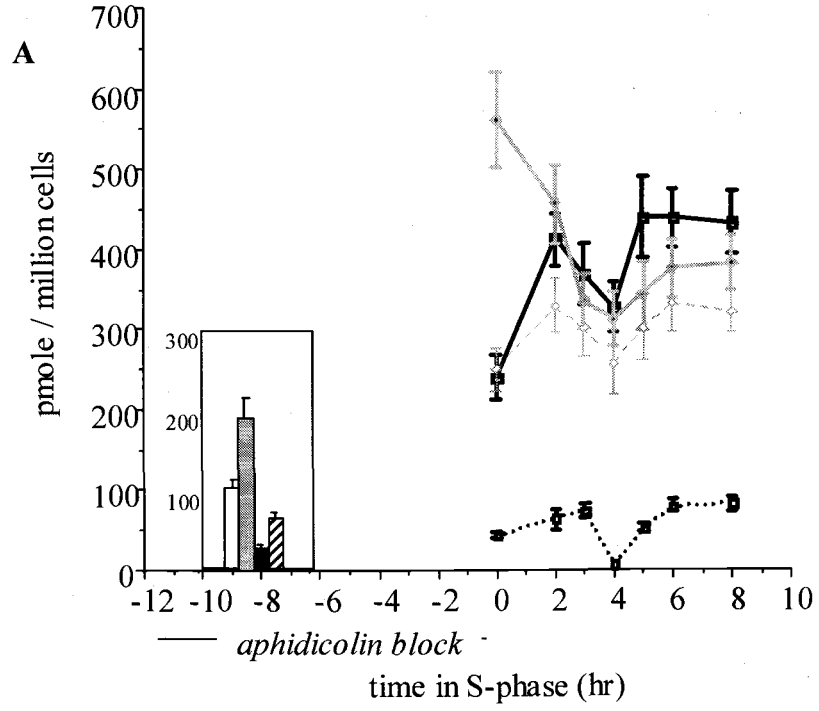
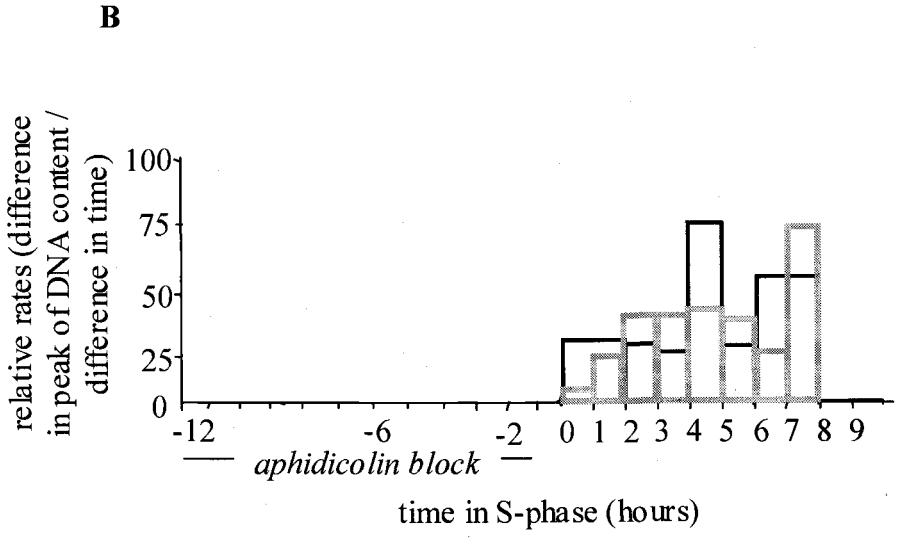

dATP
dTTP
dGTP
dCTP
pmole / million cells
time (hours) in S-phase

Figure 3.8. S-Phase dNTP Pools and DNA Accumulation Rate in HeLa Cells Synchronized with 12 Hours Aphidicolin Block. A. All four dNTP pools showed a decrease in the middle of S-phase. However, the decrease in the dGTP pools is appreciable since at the lowest concentration it can potentially affect the DNA replication rate. dATP is represented by solid black line, dTTP solid grey line, dCTP grey dotted line, and dGTP black dotted line. Insert represents the steady state HeLa cells' dNTP pool level. Open bar is dATP, grey bar dTTP, black bar dGTP and striped bar is dCTP levels. **B.** The average of DNA accumulation rate corresponds to the time points when dNTP pool levels in cells were measured. Black open bars represent the earlier measurements, and grey open bars the later measurements of DNA accumulation rates.


A
pmole / million cells
700
600
500
400
300
200
100
0
300
200
100
-12 -10 -8 -6 -4 -2 0 2 4 6 8 10
aphidicolin block
time in S-phase (hr)


B
relative rates (difference in peak of DNA content / difference in time)
100
75
50
25
0
-12 -6 -2 0 1 2 3 4 5 6 7 8 9
aphidicolin block
time in S-phase (hours)

rates were observed in early and middle stages of S-phase, they did not seem to correspond to the low dNTP pool levels (Figure 3.8). When the dNTP pool levels were averaged as early (0-3 hours), middle (3-6 hours) and late (6-8 hours) after onset of S-phase, and compared with the corresponding average DNA accumulation rates for those times, there seems to be a better correlation between the two parameters (Figure 3.9A and B). However, these averaged values were not detailed enough to give the specific time points in S-phase where dGTP might be limiting. Therefore, it still cannot be concluded that the drop in the dGTP pool level in the middle of S-phase correlates with slower rate of DNA accumulation in the middle of S-phase. It seems consistent also to note that the patterns of DNA accumulation were not just linear with slow DNA accumulation in early S-phase and highest DNA accumulation in very late S-phase, although the average DNA accumulation rates for 0-3, 3-6 and 6-8 hours of S-phase seem to show this pattern (Figure 3.9B). There was a slow DNA accumulation rate phase in middle or middle-late S-phase before the high DNA accumulation rate in late S-phase in each experiment. Can an increase in dGTP pool level inside the cell increase the DNA accumulation rates when they are relatively slow, *i.e.*, in early and middle-late S-phase (Figure 3.8B grey open bars, at hours 2 and 7 after the onset of S-phase)?

To answer the above question, the whole-cell dGTP pool during S-phase in HeLa cells needs to be increased. Addition of 2'-deoxyguanosine together with 8-aminoguanosine or guanine to the cell culture media has been reported to increase dGTP pool level in cells (Arecco *et al.*, 1988). Shown in Figure 3.10 is the result of addition of either 2'-deoxyguanosine and 8-aminoguanosine (a nucleoside phosphorylase inhibitor) or guanine to HeLa cell medium for 30 or 60 minutes before dNTP pool extraction. Addition of 2'-deoxyguanosine together with 8-aminoguanosine did increase the whole-cell dGTP pool by about two-fold. However, it also decreases the dCTP pool by more than half and dATP and dTTP pool by about 30-40%. The dNTP pools of HeLa cells after treatment with 2'-deoxyguanosine and 8-aminoguanosine are presented in Table 3.2.

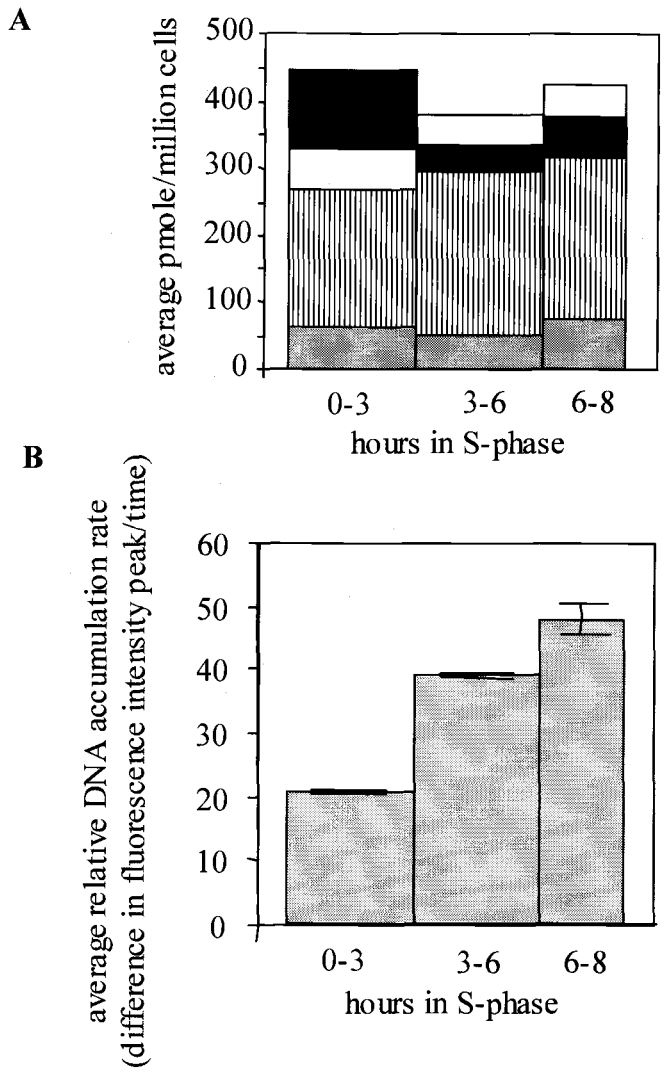


Figure 3.9. Average dNTP Pool Levels and DNA Accumulation Rates for 0-3, 3-6 and 6-8 Hours after S-Phase. A. The dNTP pool levels from Figure 3.8A are plotted to give an average at early, middle and late S-phase. Grey bars represent dGTP, striped bars dCTP, white bars dATP and black bars dTTP. **B.** Relative DNA accumulation rates from Figure 3.8B are averaged and plotted. Error bars represent the average of the two measurements made from the earlier and later experiments.

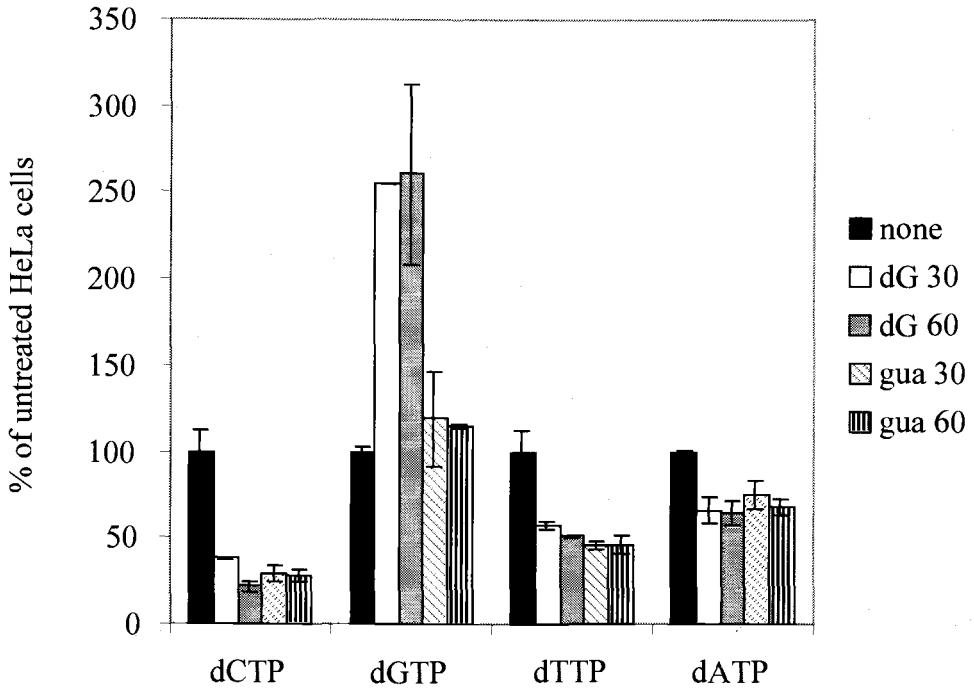


Figure 3.10. The Effect of Addition of 2'-Deoxyguanosine plus 8-Aminoguanosine and Guanine to the Whole-Cell dNTP Pools of HeLa Cells. The different drugs were added to the log-phase growing HeLa cells for the indicated amount of time (30 refers to 30 minutes incubation and 60 is 60 minutes incubation), and dNTP pools were extracted and assayed from treated and untreated HeLa cells. Error bars represent average values of two experiments.

Table 3.2

dNTP Pools of HeLa Cells Treated with 2'-Deoxyguanosine and 8-Aminoguanosine

HeLa cells treatment	pmole/million cells			
	dCTP	dGTP	dTTP	dATP
none	161	26	145	101
2'-deoxyguanosine + 8-aminoguanosine, 30 min	61	67	83	67

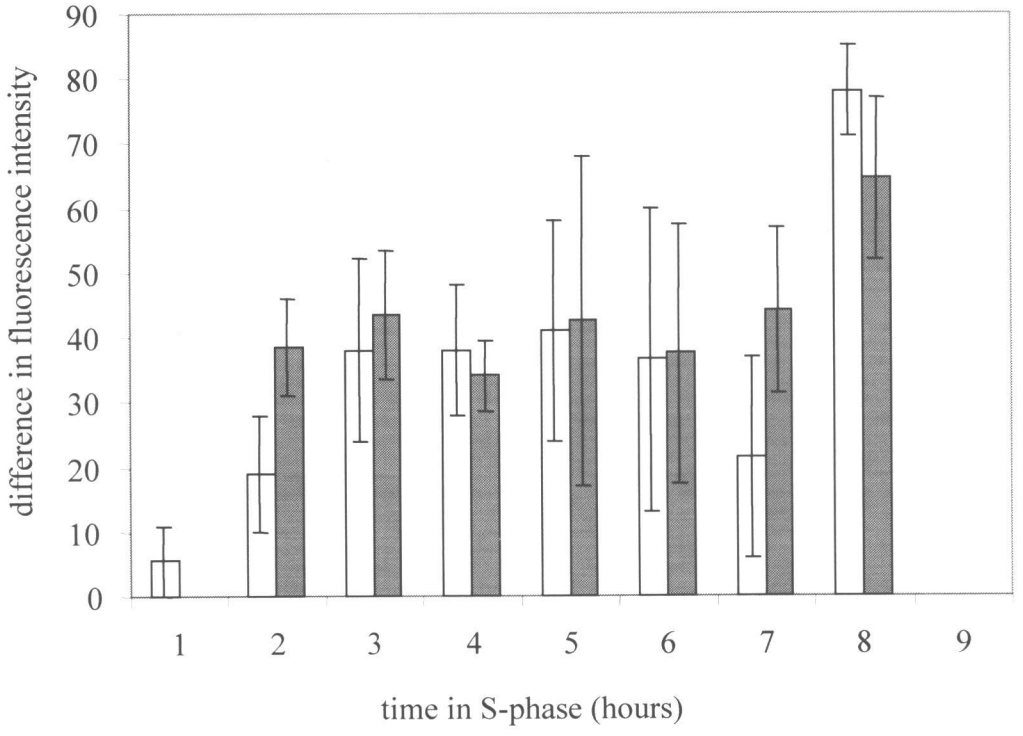


Figure 3.11. Comparison of DNA Accumulation Rates during S-Phase between Untreated and 2'-Deoxyguanosine plus 8-Aminoguanosine Treated HeLa Cells. At each time point indicated the cells were fixed for flow cytometry. The peak position representing DNA content of the population moving through S-phase in synchrony (~80% synchrony) were noted and subtracted from the peak position of the previous time point. Open bars represent untreated control and grey bars the 2'-deoxyguanosine plus 8-aminoguanosine treated cells. The error bars represent the smallest to largest differences that were seen between two sets of HeLa cell population for both the treated and untreated.

After 30 minutes treatment as described above, the levels of dCTP, dGTP and dATP are very similar. It should be noted that in the untreated control, the level of dCTP is very high compared to previously reported dCTP level from HeLa cells (see Figure 3.8A, insert). This higher than expected dCTP level has been seen occasionally for

normal growing HeLa cells in culture (personal observation); however, the reason for the high dCTP level that was observed from time to time was not clear.

Next, DNA accumulation rates of HeLa cells treated with 2'-deoxyguanosine and 8-aminoguanosine for 30 minutes before each time point were analyzed and compared with the untreated controls. As shown in Figure 3.11, from hour 3 to 6 in S-phase and also very-late in S-phase (hour 8), there did not seem to be differences in DNA accumulation rates between untreated HeLa cells and 2'-deoxyguanosine plus 8-aminoguanosine-treated HeLa cells. When the rates of DNA accumulation were slow, such as at hour 2 and 7 of S-phase (Figure 3.11), there seemed to be a possibility that an increase in dGTP level in cells from 2'-deoxyguanosine plus 8-aminoguanosine treatment could increase the DNA accumulation rates. However when the *t* -test with null hypotheses stating there were no differences between the two means of DNA accumulation rates on both hour 2 and 7 of S-phase was performed on the data shown in Figure 3.11, the *p* values were large enough so that the null hypotheses could not be rejected at 95% significance level ($0.1<p<0.05$ for the difference in the means at hour 2 of S-phase, and $0.3<p<0.25$ for the difference in the means at hour 7 of S-phase). The data do show that there were large uncertainties for the differences in DNA accumulation rates, which could mask smaller differences between the two sets of data. A repeat experiment with a higher degree of freedom (three replicates for each time points, for example) or higher degree of HeLa cells synchrony could potentially gave a cleaner data where relatively small differences between the means could be statistically significant.

DISCUSSION

Analysis of dNTP pools during S-phase in HeLa cells showed that there was no considerable change in the ratio of the four dNTPs throughout S-phase as seen in

CHO cells. This analysis was based on HeLa cells synchronized with one 12-hour aphidicolin block.

Analysis of the effect of aphidicolin on HeLa cells' dNTP pools showed that dATP and dTTP pools did accumulate to more than 200% upon prolonged exposure to aphidicolin (up to 24 hours). However, it has been shown previously that dNTP pools during S-phase would be higher than in other phases of the cell cycle. The levels of enzymes of dNTP synthesis such as ribonucleotide reductase are known to be up-regulated in late G1 phase. Moreover, dNTP pool levels during S-phase in cells synchronized with either 13- or 20-hour aphidicolin block showed only an initial elevation in the cells synchronized with 20-hour aphidicolin block. The levels of dNTP pools during the remainder of S-phase in cells synchronized with 20-hour aphidicolin block were similar to those synchronized with 13-hour aphidicolin block. The DNA content profiles at 0, 3 and 6 hours time points for both sets of cells also indicated that there were no differences in DNA accumulation rates between the cells in the two synchonization procedures.

Addition of aphidicolin back to the medium during S-phase suggested that regulation of dNTP levels by their production and turnover rate might not be similar in all the phases of S-phase.

The levels of all of the dNTP pools seem to show a decrease sometime in the middle of S-phase in HeLa cells. Very low dGTP concentration in the middle of S-phase was also observed. DNA content profiles of the cells at each time point in S-phase can be deduced to give an average DNA accumulation rate at specific time points during S-phase. However, the DNA accumulation rates did not correspond well with the dGTP levels. A repeat experiment approximating nuclear dNTP levels in parallel to DNA accumulation rates during S-phase is needed to ascertain the relation between dNTP levels and replication rates.

Despite the lack of correlation between the dGTP pool levels and the DNA accumulation rates during S-phase presented in this study, it is interesting to note that when the dGTP levels in HeLa cells were increased, and the DNA accumulation

rates were measured, there were tendencies to suggest that the increase in dGTP level, even when other dNTP levels were decreased, increased the DNA accumulation rates at times in S-phase where DNA accumulation rates had been slow in the untreated HeLa cells. Recently, Malinsky *et al.* (2001) has shown some direct evidence of the relationship between dNTP levels in cells and replication rates. They concluded that addition of exogenous dNTP increased the replication fork rates in early stage of S-phase but not in the late stage of S-phase. However, they synchronized HeLa cells in late G1 using thymidine block which is known to decrease dCTP pools in cells. Therefore, in this study the effect of the synchronization procedure on dNTP pools might have obscured the result that was obtained. In contrast to Malinsky *et al.* (2001), the result presented in this study could suggests that an increase in dGTP alone, and not in other dNTP levels, could possibly increase the slow DNA accumulation rates. Although more experiments are needed, this result could indicate the first evidence of the potential role of dGTP in S-phase DNA replication rates in mammalian cells.

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CHAPTER 4

INDICATION OF SPONTANEOUS MUTATION RATE VARIATION DURING MAMMALIAN CELL CYCLE

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SUMMARY

A *gfp* gene carrying an amber mutation in amino acid position 39 (*gfp-amb*) was inserted in different regions of HeLa cells' chromosomes that are replicated either early, middle or late in the S-phase. Each HeLa cell clone was grown to reach high enough population number to generate spontaneous mutation in one generation, and then the mutant cells that had arisen, *i.e.,* cells displaying green fluorescent phenotype, were sorted out from the population to eliminate the background mutants in each clone. Two clones were designated middle-replicating and late-replicating *gfp-amb* through both fluorescent *in situ* hybridization and a quantitative PCR method. The middle-replicating *gfp-amb* HeLa clone always accumulated more mutants than the late-replicating clone after four days in culture. The spontaneous mutation rate of the late-replicating clone was found to be at least two-fold higher than the middle-replicating clone. This finding corresponds with dNTP level modulation during S-phase in HeLa cells; dNTP levels are lower in middle S-phase. Previous experiment using *in vitro* replication reactions showed that replication with lower dNTP levels resulted in higher replication fidelity. These data are consistent with the hypothesis that spontaneous mutation rates vary within the S-phase, and that replication rate and dNTP level changes may be responsible for this variation.

INTRODUCTION

Differences in spontaneous mutation rate of early and late replicating genes were first proposed as an explanation for the origin of isochores (Gu and Li, 1994), *i.e.,* regions in mammalian chromosomes that have compositionally homogeneous GC contents (reviewed in Sabeur *et al.*, 1993). Gu and Li (1994) proposed a kinetic model in which changes in deoxyribonucleoside triphosphate (dNTP) concentrations thoughout S-phase in mammalian cells as shown for Chinese Hamster Ovary (CHO)

cells (Leeds *et al.*, 1985) would lead to different mutational biases at different regions of the chromosomes. The mutational bias could explain the existence of isochores if each isochore region were replicated at a specific time during S-phase. However, replication of a specific isochore was not always exclusively either early or late in S-phase. Eyre-Walker (1992) noted that some GC-rich and -poor isochores were replicated both early and late in the cell cycle. Moreover, the *in vitro* DNA replication experiments showed that the fidelity of replication did not change significantly with changes in relative dNTP pool concentrations supplied for replication (Chapter 2).

The *in vitro* DNA replication experiments suggested that higher concentrations of each dNTP, even without any changes in the ratio of each dNTP, affected DNA replication fidelity more than a deficiency of one dNTP during replication (Chapter 2). It is interesting that in aphidicolin-synchronized HeLa cells, the concentrations of all four dNTP pools during S-phase seemed to vary temporally (Chapter 3). However, the relationship between measured dNTP pool and "replication active" pools, the dNTP pool that is seen by DNA polymerases in the different replication foci during DNA relication in mammalian cells is poorly understood (reviewed in Mathews and Ji, 1992). Nonetheless perturbations of dNTP pools have been documented to produce a range of genetic effects associated with inaccurate DNA replication and repair (reviewed in Kunz and Kohalmi, 1991).

Temporal differences in DNA replication rates from mammalian cells in culture have also been observed (Collins *et al.*, 1980). In HeLa S3 cells, there seemed to be three phases of DNA accumulation during S-phase: *i.e.,* early fast phase, slower middle phase and faster late phase (Collins, 1978). Our observation showed that HeLa cell dNTP pools varies during S-phase. Moreover, a recent report has indicated that addition of exogenous dNTP could accelerate the speed of replication fork movement in early S-phase in HeLa cells (Malinsky, *et al.*, 2001). This observation directly relates dNTP concentrations during S-phase and DNA replication rate.

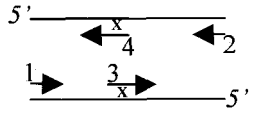
The use of green fluorescent protein (GFP) to evaluate spontaneous mutation rate as described by Bachl *et al.,* (1999) have been adapted to the study reported here. GFP is chosen as the reporter for mutation in cells because cells that display green fluorescent phenotype can be either sorted-out from the population or collected; hence, the starting populations for spontaneous mutation rate measurements are free from background mutant cells. Different number of mutant cells that could arise during growth can lead to very significant differences of calculated mutation rates between replicate cultures when they are not eliminated.

The spontaneous mutation rates of GFP-transfected HeLa cell clones that have the *gfp* gene inserted in temporally different locations in the chromosomes: *i.e.*, early, middle or late in S-phase were compared to examine whether there is a measurable difference in spontaneous mutation rates between stretches of DNA replicated early and late in S-phase in mammalian cells.

MATERIALS AND METHODS

CONSTRUCTION OF PLASMID ENCODING gfp-amber GENE

Plasmid pEGFP-C1 encoding the green fluorescent protein (GFP) with mutations from Phe64 to Leu and Ser65 to Thr as well as silent mutations to use the human preferred coding sequences was obtained from Clontech Laboratories. Site directed mutagenesis by PCR was used to obtain pEGFPamb-C1. Primers: 1 (GFP-AM1), $^{5'}$*GCT ACC GGT CGC CAC*; 2 (GFP-AM2), $^{5'}$*GCT CGA GAT CTG AGT CC*; 3 (GFP-AM3), $^{5'}$*GAT GCC ACC TAG GGC AAG C*; and 4 (GFP-AM4), $^{5'}$ *GTC AGC TTG CCC TAG GTG G* were used as diagrammed to yield a tyrosine to amber mutation at codon 39 of the GFP. Mutagenesis by four-primer PCR was done as described in (Ho *et al.,* 1989).



Primers 1 with 4 and 2 with 3 were used with the pEGFP-C1 template separately to amplify two different segments of the gene and introduce the mutated DNA codon into the fragments. Next, the two amplified segments were used as template in a PCR reaction, and primers 1 and 2 were used to amplify the mutated gene. The PCR program used was as follows: first cycle melting temperature was at 95° C for 5 minutes, annealing at 50° C for 1 minute and extension at 72° C for 1 minute; the subsequent 20 cycles melting temperature was at 95° C for 1 minute, annealing at 50° C for 1 minute and extension at 72° C for 1 minute; the last cycle melting temperature was at 95° C for 1 minute, annealing at 50° C for 1 minute and extension at 72° C for 5 minutes. The resulting *gfp-amb* gene was selected as having a new *Avr*II (New England Biolabs) restriction site in the gene. Plasmid pEGFPamb-C1 was constructed by replacing the *gfp* gene cassette with the *gfp-amb* gene cassette. After transformation into *E. coli* XL-1 Blue, pEGFPamb-C1 DNA isolated from three different clones that tested positive for the presence of the new restriction site in the plasmid was sequenced to confirm the presence of the mutated codon and the absence of any other mutation. Sequencing was done in the Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR.

TRANSFECTION AND SELECTION OF HELA CLONES CONTAINING gfp-amb GENE

Electroporation was chosen as the method for introducing pEGFPamb-C1 DNA into the HeLa cells. Stable transfection using electroporation, as reported, usually resulted in incorporation of one copy or more rarely two or three copies of the DNA per cell (Potter, 1996). As noted, stable transfection with other methods

such as lipofectin (Life Technologies), or calcium chloride resulted in tandem incorporation of the DNA into a site in the host chromosome. Usually tandem incorporation could result in up to 40 copies of the gene of interest per cell. For our study, it is important to have just one copy of the *gfp-amb* gene per cell in each clone for valid comparison of spontaneous mutation rate between the clones. Therefore, electroporation was chosen as the transfection method.

Electroporation done at 250 V using a BTI electroporator yielded an optimal result in the proportion of cells that took up the DNA *versus* the cells that died during the process. Before electroporation, the cells were trypsinized, washed two times with ice-cold sterile phosphate-buffered saline (PBS: 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4 in 1 L double-distilled H_2O (ddH_2O), prepared as 10x solution) and resuspended at a concentration of 10^7 cells/ml for stable transfection and 10 x 10^7 cells/ml for transient transfection. Linearized DNA was used for stable transfection and circular plasmid DNA was used for transient transfection. pEGFPamb-C1 was linearized with restriction enzyme *Dra*III (New England Biolabs). Linearizing the plasmid with this enzyme was found to give a higher proportion of stable transfectants that carry the neomycin gene for drug selection as well as the complete *gfp-amb* gene. Ten μg of linearized DNA per 100 μl of cell suspension was used for stable transfection, while 25 μg of plasmid per 100 μl of cell suspension was used for transient transfection.

The HeLa cells were maintained in 1:1 DMEM/F-12 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS). Geneticin, G418 (Roche Molecular Biochemicals) was used as the selection for the transfected cells. 700 μg/mL G418 in the medium was found to prevent untransfected HeLa cell growth; therefore, this concentration of G418 was used in the media for selection of clones and also for maintaining each clone in culture.

For stable transfection, the electroporated cells were plated at limiting dilution in 24-well plates. The content of each well was calculated from previous experiment to yield about 2-4 stable clones. Each clone was trypsinized inside a

cloning ring and was replated and grown until they reached a population size big enough (~10^6 cells) for DNA extraction followed by PCR screening. For transient transfection, the electroporated cells were plated onto 2 100-mm plates and grown for 48 hours without any selection. The cells then were harvested and ready for experiments.

PCR primers used for screening were: GFP-1A, $^{5'}$ *GCA GAG CTG GTT TAG TGA ACC G* and GFP-2A, $^{5'}$ *CGT CGA CTG CAG AAT TCG AAG C* that would amplify a complete *gfp-amb* gene only out of total cellular DNA under PCR conditions used. The PCR program was as before except that the annealing steps were done at 68° C. These primers did not yield any PCR amplification when used in the reaction with DNA extracted from untransfected HeLa cells. Total DNA template of at least 0.5 μg per 100 μL reaction and 25 cycles were needed in order to see any PCR amplification.

SOUTHERN BLOTTING

Eighty μg of isolated genomic DNA each was used per clone to detect the presence of the *gfp-amb* gene. The DNA was divided into four restriction digestion reactions, 20 μg of DNA each, and it was digested with 5 X excess of enzyme unit (5U/μg of DNA) to ensure complete digestion. Afterwards, the reaction mixtures were extracted with phenol-chloroform once and precipitated with isopropanol and 3 M NH_4Ac. The DNA was collected through centrifugation, pooled together in 40 μL of TE and loaded onto the prepared 0.5 X TBE-agarose gel with ethidium bromide that had been casted to have wide wells to accommodate the volume of the sample. The gel was run in 0.5 X TBE buffer at 20 V overnight. The gel was then depurinated by immersing in 0.25 M HCl for 10 minutes with shaking. Immediately afterward the gel was rinsed in ddH_2O twice. Before Southern transfer, the gel was denatured in 0.5 M NaOH, 1.5 M NaCl for 30 –60 minutes at room temperature with

shaking and was neutralized in 1.0 M Tris-HCl (pH 8.0), 1.5 M NaCl for another 30-60 minutes at room temperature with shaking. The DNA was blotted from the gel by capillary transfer to a positively charged membrane (Magnacharge, Micron Separations Inc.) using 10 X SSC (made as 20 X SSC stock, 3 M NaCl, 300 mM Na Citrate pH. 7.0) buffer overnight.

The membrane was washed in 5 X SSC buffer for 1 minute at room temperature and air-dried. It was then UV crosslinked to fix the DNA to the membrane. The membrane was then prehybridized in 50 mL of 5 X SSC, 2% blocking reagent for nucleic acid hybridization from the Genius System (Boehringer Mannheim), 0.1 N-lauroylsarcosine, 0.02% sodium dodecyl sulfate (SDS) and 50% deionized formamide (50% deionized formamide, 3 X SSC, 10 mM EDTA (pH. 7.2), 0.06 M sodium phosphate buffer, 3 X Denhardt's solution, 0.2% SDS has worked well also) at 37° C for 3 hours. Afterward, the probe mixture was added to the solution, and the incubation was allowed to proceed for another 20 hours.

The probe was made with random oligonucleotide priming method, using heat denatured *gfp* gene as the template, [α-^{32}P]dATP and Klenow fragment, in 20 μL reaction mixture incubated at 37° C for 4-6 hours. The reaction mixtures were diluted to 100 mL with TE buffer, and it was processed through a desalting column (Centri-Sep, Princeton Separations) to remove all the free nucleotides. Five μL of the reaction was counted (Cerenkov count), and a calculated amount of at least 1 x 10^7 cpm/mL was used in the hybridization reaction. The calculated amount of probe and 50-100 μL of 10 mg/mL carrier DNA in a total volume of 400 μL was added to 50 μL of probe denaturing solution (1.2 M NaOH, 12 mM EDTA). The mixture was mixed, centrifuged to bring the solution to the bottom of the tube, placed in a 65° C water bath for 5 minutes and immediately put into ice before adding it to the prehybridization solution.

The membrane was washed in a series of large volume washes. Immediately after hybridization, the membrane was washed quickly with room temperature 0.1 X SSC, 0.2% SDS twice in the hybridization dish. It was washed twice more in two

large glass trays with about 200 mL each of the same solution. Then the membrane was incubated twice in a large tray with rocking with approximately 400 mL of the same solution for 1 hour each at 37° C with two washes in between. The last wash was done with 5 mM EDTA (pH 7.2), 0.2% SDS for another hour at 37° C. The membrane was then rinsed with room temperature 0.1 X SSC, air-dried and placed on the phosphoimager screen between Saran wrap layers.

DETERMINATION OF REPLICATION TIMING USING FLUORESCENCE IN SITU HYBRIDIZATION

Determination of replication timing of single copy genes with fluorescence *in situ* hybridization (FISH) was according to the method of Selig *et al.* (1992) and Ausubel *et al.* (1989) with some modification. Briefly, the cells were grown for 20 hours on sterile glass slides fitted with detachable media chambers. The experiment was started by washing the cells attached to the slides in ice-cold PBS twice. After, 4% paraformaldehyde (PFA) fixative (prepare immediately before use by heating the 4% PFA solution in 2/3 of the ddH_2O volume needed to 60° C and adding 1 drop of 2 N NaOH; after the solution is almost clear, add concentrated PBS enough to make 1 X and adjust pH to 7.2 with concentrated HCl; filter sterilize; cool to room temperature) was added to cover the cells which were then incubated in the solution for 20 minutes at room temperature. After incubation, the fixative was aspirated, 3 X PBS was added to each slide chamber, which was then incubated for 2 minutes to stop the fixation. The slides were washed with 1 X PBS twice for 2 minutes each. After the PBS washes, the detachable chambers were removed, and each slide was dehydrated through a series of 5-minutes incubation in 50%, 70%, 95% and 100% ethanol which were done in 150-mm tissue culture dishes. The slides were then air-dried completely and transferred to slide boxes containing desiccant to be stored for at least overnight at –80° C.

The next day the slides were prewarmed to room temperature from –80° C before they were rehydrated through a series of 2-minutes 100%, 95%, 70% and 50% ethanol incubations. Next, the slides were denatured in 0.2 M HCl for 20 minutes at room temperature and for 15 minutes at 70° C in 70% deionized formamide, 2 X SSC. Next, the slides were rinsed in 1 X PBS for 2 minutes, fixed with freshly prepared 4% PFA fixative for 5 minutes at room temperature, rinsed again with 3 X PBS to stop the fixation and washed twice, 30 seconds each in 1 X PBS. The slides were dehydrated through a series of ethanol washes again, 2 minutes each, first in 50%, then 70%, 95%, 100% and another 100% ethanol. After they were completely air-dried at room temperature, the slides were put in a box with desiccator and left at –80° C overnight.

The probe mixture was digoxigenin-labelled DNA that was made using the Genius kit (Boehringer Mannheim) according to the manufacturer's instruction. Briefly, DNA template was made from *Bgl*II and *Bsh*TI (MBI Fermentas) double digest of pEGFP-C1 that cut the *gfp* gene cassette from the plasmid. The digest was analyzed in an agarose gel, and the *gfp* gene band of interest was cut from the gel and purified with a gel extraction kit (BioRad) according to manufacturer's instruction. The yield was quantitated using UV absorbance at 260 nm, and the DNA was used as template in the labelling reaction. The labelling reaction was done using 20 ng of denatured template DNA. The template was denatured by incubating an Eppendorf microcentrifuge tube with template DNA inside in a boiling water bath for 10 minutes, then the tube was immediately put into liquid nitrogen to cool as fast as possible. The labelling reaction was done as suggested by the Genius kit instruction with the denatured template DNA, random hexanucleotide primers (Promega), dNTP mixtures with digoxigenin-labelled dUTP (Genius kit vial #6) and DNA polymerase I (Klenow fragment) (USB) in 20-µl total reaction mixture at 37° C overnight. Estimation of yield was done using alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche Molecular Biochemicals), and usually about 50 ng/µl of digoxigenin-labelled DNA was obtained.

For fluorescence *in situ* hybridization, the probe and the non-specific salmon sperm DNA need to be of 250 base pairs or less in length (Selig, 1992). The reaction mixture (50 μL) for each slide containing 10 μg of sonicated salmon sperm DNA (Pharmacia) was digested with DNAse I (0.5 mgl/ml) in React4 buffer (Promega) for exactly 4 minutes at room temperature. After adding 50 ng of DNA probe, the reaction was allowed to proceed 1.5 more minutes before 25 μl of 0.25 M EDTA was added to stop the reaction. The resulting DNA fragments were of 100-300 base pairs in length as confirmed by 1.2% agarose-TBE gel electrophoresis. After digestion the DNA in the mixture was precipitated with EtOH-NH_4Ac, and the pellet was resuspended in 10 μl of 50% deionized formamide, 1 X SSC, and 3 X Denhardt solution (100 X stock solution: 2 g of BSA, 2 g of PVP, 2 g of Ficoll in 100 mL solution). The DNA was denatured by incubation in a 70° C water bath for 10 minutes. Then it was immediately put on ice until the mixture was put onto the prepared slide for hybridization.

The slides were brought to room temperature, prewarmed to 37° C before 10 μL of the probe mixture was applied, then the cover slip was mounted and sealed with 2% agarose. The slide was put into a moist chamber (moist chamber solution has the same formamide concentration as the probe mixture) and incubated at 37° C for overnight.

After hybridization, washing was done according to Lichter *et al.* (1988) with some modifications. Briefly, the slide was washed three times, 30 minutes each in 50 mL of 50% formamide 2 X SSC at 42° C. Then it was washed three times, 30 minutes each in 50 mL of 0.05 X SSC at 42° C.

The bound digoxigenin detection was done with Fluorescent Antibody Enhancer Set for DIG Detection kit (Boehringer Mannheim) as described in the manufacturer's instructions. After washing, the slide was washed briefly with the blocking solution (1:10 dilution of supplied blocking solution (vial #4) in PBS), and each slide was put in a 50-ml conical polypropylene tube with 30 ml blocking solution at room temperature for 30-60 minutes. Then the slide was drained on a

paper towel, and 2 μl of first antibody (anti-DIG monoclonal Ab against digoxigenin, mouse IgG1) in 25 μl blocking solution was added onto the cells. Next, a cover slip was put on top and the slide was put inside a moist chamber at 37° C for 1 hour. Afterward, the slide was washed three times briefly with PBS-Tween (0.02 % Tween in 1 X PBS) wash solution at 37° C. The second antibody (anti-mouse-Ig-DIG, $F(ab')_2$ fragment) was applied, and again the slide was incubated in moist chamber at 37° C for 1 hour. After washing as before, the third antibody (anti-DIG-Fluorescein, Fab fragments) was applied in the dark. The slide was incubated in the moist chamber at 37° C, for 1 hour in the dark. All the manipulations done on the slide after this point were done in the dark. The slide was washed three times, five minutes each at 37° C with the PBS-Tween washing solution. After air-drying, the slide was washed several times briefly with PBS solution. It was then counterstained with either propidium iodide (1 μg/ml) in PBS or Hoechst stain in PBS for five minutes. An antifade solution, Prolong (Molecular Probes), was applied according to manufacturer's instruction. A coverslip was then mounted on the slide and sealed with clear nail polish the next day. The slide was viewed using a fluorescence miscroscope (Zeiss Axiovert S 100 TV) with 100 X magnifications with oil immersion and also a zoom lens (Dr. Jeff Greenwood, Environmental Health Sciences Center, Cell Culture Facility at Oregon State University).

DETERMINATION OF REPLICATION TIMING WITH QUANTITATIVE PCR

The cells were synchronized using aphidicolin. Aphidicolin (3μM) was added to the log-phase growing cells for 20 hours. This was followed by washing and addition of medium without aphidicolin for 15 hours and again addition of aphidicolin to the medium for another 20 hours (as described in Chapter 3). At each time point the cells were trypsinized, washed with ice cold PBS and resuspended in digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8), 25 mM EDTA pH 8, 0.5%

SDS, and 0.1 mg/ml proteinase K added fresh for each use). The digestion was allowed to continue overnight at 42° C. The DNA was phenol/chloroform-extracted and precipitated with ethanol/ ammonium acetate (EtOH/NH_4Ac) method. It was immediately centrifuged in a fixed-angle Eppendorf microcentrifuge at 8000 rpm for 4 minutes. The pellet was washed with ice-cold 70% ethanol, and resuspended in TE. Usually the pellet was not readily resuspended into the solution, and it might need to be incubated at 37°C for a couple of hours. After the pellet was dissolved, the concentration of DNA in the solution was determined, and 0.6 μg of DNA was used as template for the PCR reaction.

PCR was done in 100-μl total reaction mixture. The same primers as the ones used for screening the clones were used with annealing temperature also at 68° C. 5U of Taq DNA polymerase was used per 100-μl reaction. Ten mL of the resulting PCR reaction was loaded into the well of 1 % agarose-TBE gel stained with ethidium bromide. After the tracking dye had run to the middle of the gel, the gel was visualized using a UV-lightbox. Pictures were taken, and the band intensities were analyzed using ImageQuant Image analysis software.

DETERMINATION OF SPONTANEOUS MUTATION RATES

A Fluorescence-Activated Cell Sorter (FACS) was used to purge the cell population from already arising mutants that would complicate the analysis of spontaneous mutation rate per clone. Each clone was grown to approximately 200 million cells and harvested. Trypsinization was stopped with 100% FBS instead of medium containing 10% FBS. The cells were washed twice with ice cold sterile PBS and resuspended in about 3 ml of PBS.

The cells were analyzed with a high speed MoFlo (Cytomation, Inc., Fort Collins, CO) FACS machine courtesy of Dr. Steven Giovannoni, Dept. Microbiology, Oregon State University. The fluorescence gating was determined by

running a two-way sort of pEGFP-transiently transfected HeLa cell population. The phenotype of each sorted population was confirmed with a fluorescence microscope. Usually the pEGFPamb-C1 HeLa clonal populations were more than 99% nonfluorescent; *i.e.,* mutations to fluorescence phenotype did not accumulate in these cells. The recovery of the cells after the sorting process was usually very low, in the order of 10% from the original cell number harvested.

After sorting, depending on the calculated number of the sorted cells, the cells were plated onto one or two 150-mm tissue culture plates (Fisher). A quarter of the sorted cells could be used for flow cytometry to confirm the result of the sorting process. At the indicated time point (days) the cells were trypsinized, one half of the cells population were taken for flow cytometry analysis, and the rest were plated back onto a new plate using the old conditioned medium.

RESULTS

HELA CELL CLONES STABLY INCORPORATING gfp-amb GENE

Ten HeLa cell clones resistant to G418 were chosen and tested them for the presence of *gfp-amb* gene in their genome. Total HeLa cell DNA from each clone was extracted and subjected to PCR followed by Southern blotting to detect the presence of the *gfp-amb* gene. Five clones, namely 5, 9, 10, 17 and 19 were confirmed to have the complete *gfp-amb* gene. All five clones showed only one PCR-amplified band of the approximate size (~800 bp), confirming that one copy of the gene was inserted in each location in the chromosome (Figure 4.1).

Electroporation was chosen as the method to deliver the DNA into HeLa cells, since it was reported that stable transfectants achieved by electroporation of DNA into mammalian cells usually resulted in cells having insertion of one copy of

the gene per cell. Two or three copies of the gene inserted in the chromosome are less common compared to the single-copy insertion. It was confirmed by PCR that all five clones had only one copy of the gene inserted in one location of the chromosome. Tandem insertion of the gene in one location in the chromosome would show as a ladder when the PCR product was analyzed on a gel. However, PCR method can only detect either single or tandem insertion of a particular DNA sequence in a location in the chromosome. Therefore, Southern blotting analysis was done to confirm that there was only one copy of *gfp-amb* gene per cell in each clone.

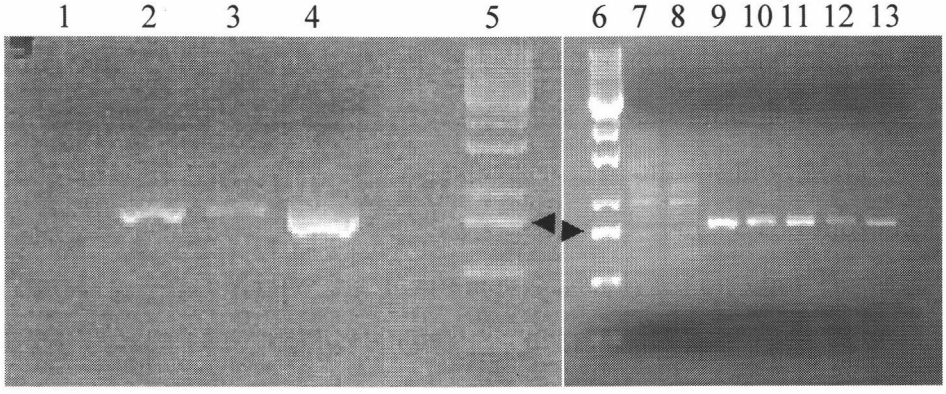


Figure 4.1. PCR Screening of HeLa Clones. Lane 1 is PCR product of the untransfected HeLa cell DNA; 2 and 3 show amplification from *gfp*-transfected HeLa cells; 4 shows PCR product from pEGFP plasmid that served as positive control; 5 and 6 show the 1 kb ladder; the next two lanes show PCR product from another set of primers that resulted in nonspecific amplification of DNA from (7) untransfected HeLa, and (8) transfected HeLa cells; and 9 through 13 are PCR products of DNA isolated from clones 5, 9, 10, 17 and 19 respectively. 750 bp on the ladders are shown by arrowheads. Ten μL of the PCR reaction each (total volume of each reaction was 100 μL) was loaded into each lane of the 1.2% agarose-TBE gel stained with EtBr. The gel was run at 100 V, for approximately 1 hour.

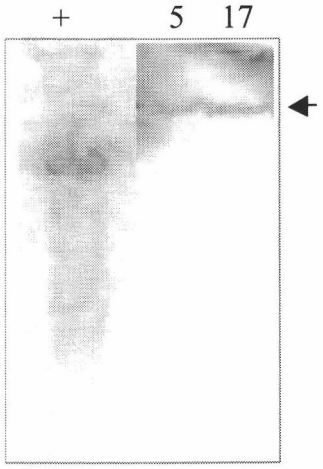


Figure 4.2. Southern Blotting Analysis of Clones 5 and 17. Only one band corresponding to *gfp-amb* gene was present in the genomic DNA from each clone (arrow). Lane '+' represents the positive control (1 ng of pEGFPamb-C1 and 80 μg of salmon sperm DNA), lane 5 and 17 represent clones 5 and 17 respectively.

The Southern analyses showed only one high molecular band for each clone chosen for further analysis, *i. e.*: 5 and 17 (Figure 4.2). However, since about 80 μg of DNA per lane was needed in order to detect the presence of the gene using the Southern blotting method, there was a possibility that the DNA was not restriction digested to completion, and there was not enough separation between the fragments. For this reason it is difficult to unambiguously interpret the result of the Southern blotting experiment.

The presence of single-copy insertion per cell per clone was confirmed later in the fluorescence *in situ* hybridization (FISH) experiment described in the next section. Under the fluorescence microscope, almost all of the cells in the clones 5 and 17 appeared to have either single or double-dots instead of double or two double-dots for before and after replication if the *gfp-amb* gene was inserted in two different locations in the chromosome. Therefore, there is no evidence to suggest that clones number 5 and 17 had more than a single insertion of the *gfp-amb* gene in their chromosomes. Clones 9, 10 and 19 were not tested further since in initial

experiments their spontaneous mutation rates were not linear or else were abnormally high. Initial spontaneous mutation frequencies for clones 10 and 19 were around 100 mutants/million cells, indicating that the gene might have increased the spontaneous mutation rates of the clones, for example by disrupting the essential gene or genes for DNA repair and replication in these clones.

DETERMINATION OF REPLICATION TIMING FOR EACH gfp-amb GENE

The *gfp-amb* gene was inserted randomly in HeLa cell chromosomes. Therefore, some clones might arise from cells that have the gene inserted in a chromosomal region that is replicated early in S-phase while other clones come from cells that have the gene inserted in the chromosomal region that is replicated late in S-phase.

Selig *et al.* (1992) have used FISH to analyze replication timing of single-copy genes in human chromosomes. Even without cell-cycle synchronization they showed that the *in situ* fluorescence hybridization pattern of an early-replicating gene in a cell population consists of more double fluorescence dots for the gene and its duplicate, indicating that more cells in the culture have completed replication of that sequence the late replicating cells would consist of more single fluorescence dot per cell indicating that the gene of interest had not replicated in most cells.

Using the method described above, the replication timing of the *gfp-amb* genes in clone numbers 5 and 17 was analyzed. *gfp* gene cut from pEGFP-C1 was used to make the digoxigenin-labelled probes which were used for the hybridization. Fluorescein-conjugated antibody cascade was used to detect digoxigenin-labelled probe bound to chromosomal DNA on the slide (see MATERIALS AND METHODS). The FISH pattern would be a single fluorescent dot per cell for the *gfp-amb* gene that had not undergone replication and double-dots for the gene that had undergone replication (Figure 4.3). Since in a log-phase growing cell population

most cells are not replicating their DNA, the majority of the cells (about 60-70%) would have the gene as a single copy and would appear to have single-dots. Therefore, the difference between the early and late replicating gene in each clones would be determined only by a small fraction of the total population. In a randomly growing cell population, a snapshot of the FISH pattern from a population of cell in which the *gfp* gene has been inserted in the genomic region that is replicated early in S-phase would consist of a higher proportions of double dots compared to cells with late-replicating gene. A snapshot of the FISH pattern from a population of cell in which the *gfp* gene has been inserted in the region that is replicated late in S-phase would consist of mostly single dots. From these data an order of where the gene has been inserted among the clones tested can be constructed.

The result of the *in situ* hybridization experiment is shown in Table 4.1. From these observations, clone number 5 most probably has the *gfp-amb* gene in the later replicating chromosomal region compared to clone 17, and clone number 17 has the *gfp-amb* gene replicated earlier during S-phase.

The replication timing of the inserted *gfp-amb* gene was also determined using a quantitative PCR method to confirm the results of the FISH experiment. It was observed that the amount of total DNA that was used for detection by PCR is relative to the intensity of the resulting band of amplified DNA for a certain concentration of template DNA (Figure 4.4). Since there was only one copy of the gene per cell in each population at large, one can compare the resulting PCR amplified band intensities of reactions using a constant DNA concentration isolated for each time point from synchronized cell populations. When the same DNA concentration is used for PCR reaction but there are two copies of the *gfp-amb* gene per cell after it is replicated, the intensity of the resulting amplified DNA is increased. By synchronizing the cell at early S-phase and extracting DNA from the cells at each point during S-phase then performing quantitative PCR, we can derive the replication timing of the *gfp-amb* gene for each clone. An increase of band

Figure 4.3. Fluorescent *In Situ* Hybridization of Clones 5 and 17 Showing Single and Double-Dots for *gfp-amb* Gene. The pictures on the right are larger versions of each picture on the left showing either the single or double-dots more clearly. *Gfp-amb* gene was detected using digoxigenin probe tagged with fluorescein, and the cells were counterstained with Hoescht stain to detect the nucleus (see MATERIALS AND METHODS).

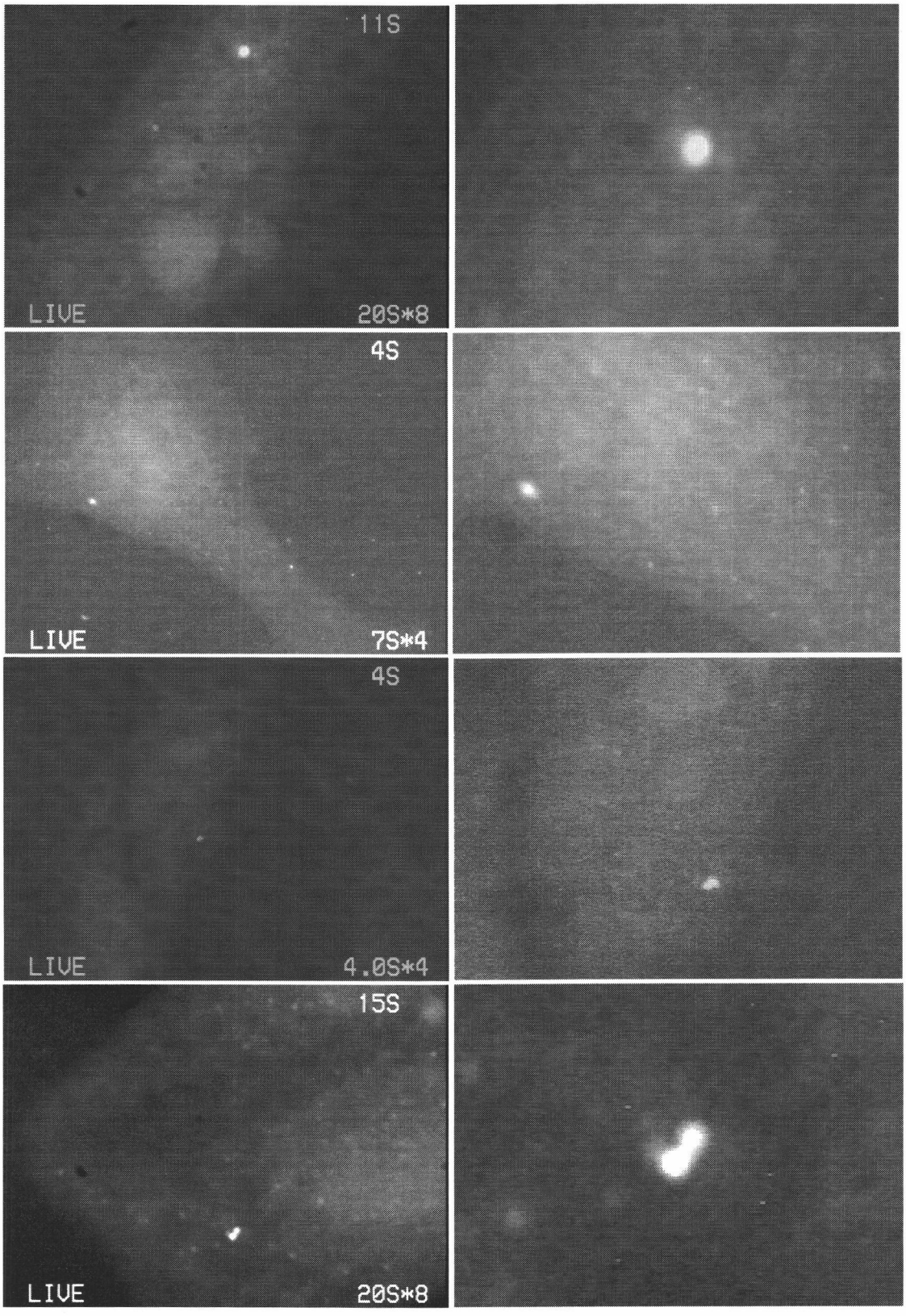

11S
LIVE
20S*8
4S
LIVE
7S*4
4S
LIVE
4.0S*4
15S
LIVE
20S*8

Table 4.1

Determination of Replication Timing with FISH Method

Clone #	Single-Dot		Double-Dots	
	Counted	Proportion	Counted	Proportion
5	247/273	0.905	26/273	0.095
17	185/217	0.853	32/217	0.147

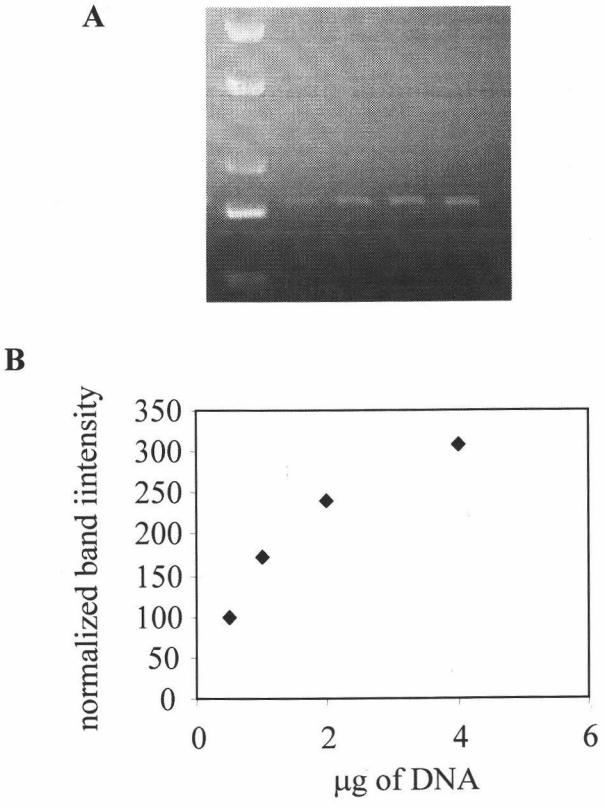


Figure 4.4. Relative Band Intensity as a Function of Total Amount of DNA used in the PCR. From total template DNA of 0.5 to 1 µg in 100 µL PCR, the relative band intensity (measured as volume in ImageQuant analysis) increased when the amplified DNA was run on 1% TBE-agarose gel as pictures in (**A**). The band intensities were quantified using ImageQuant (**B**).

intensities would represent the time in the S-phase that the gene has been replicated (pointed by the arrows in Figure 4.5).

In practice, there are a lot of factors that can affect the results of quantitative PCR experiments described above. For example, the intensity of each band depends on the concentration of ethidium bromide (EtBr) in the gel. For comparison purposes, the uniformity of EtBr concentration throughout the gel and the uniformity of UV light illumination during viewing are very important factors to ensure proper

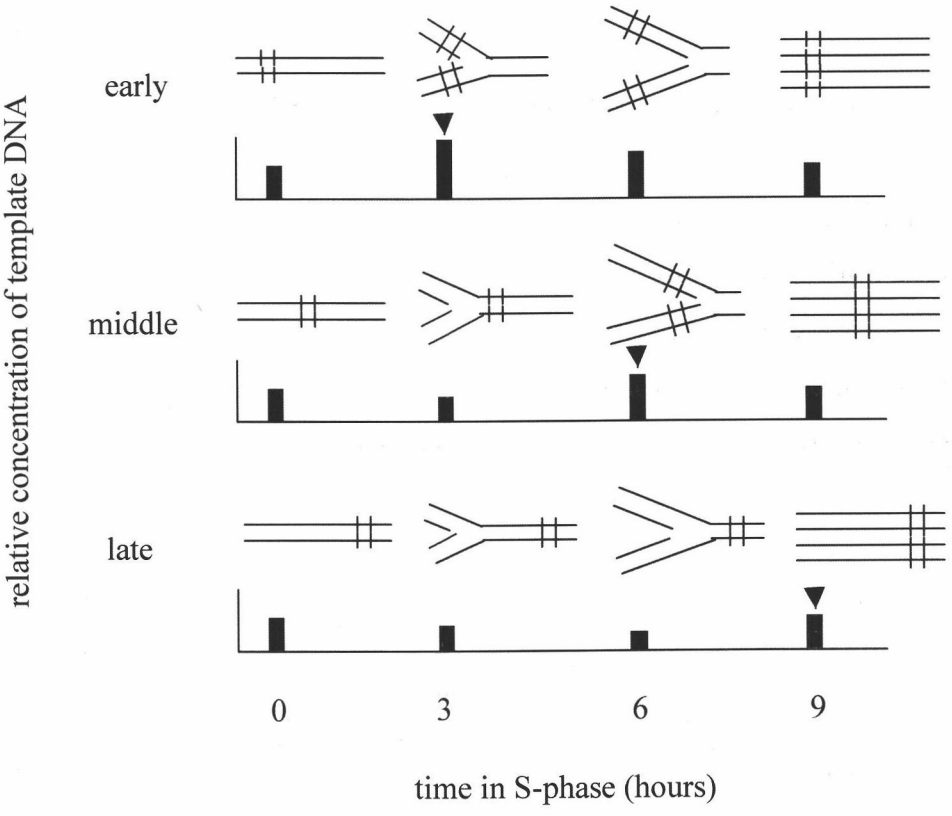
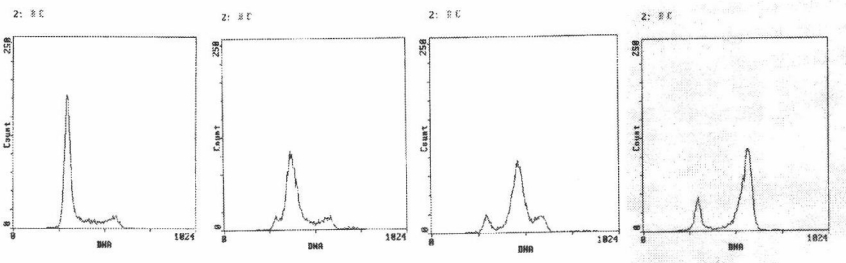


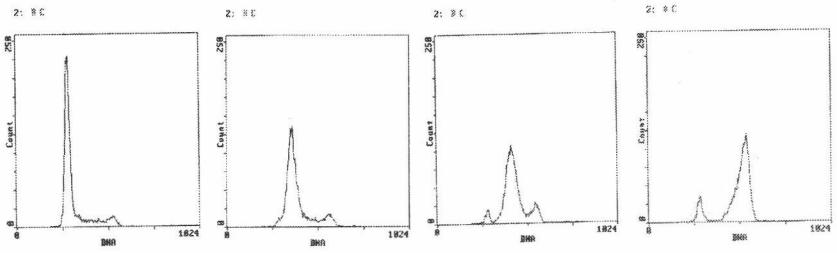
Figure 4.5. Diagram of Relative Template Concentration *versus* Replication Timing. The above diagram showed how in a single copy gene the relative template concentration varies throughout S-phase depending on when the gene is replicated. With a constant amount of isolated DNA used, relative template concentration in a PCR resulted in different band intensities, which can be used to deduce the replication timing of the gene.

Figure 4.6. Result of Quantitative PCR for Replication Timing Determination. **A**. The synchronization using aphidicolin resulted in about 80% synchrony of the cell population through S-phase. The DNA content profiles for 0, 3, 6 and 10 hours after S-phase are shown from left to right for each clone. The resulting PCR bands (**B**), also from 0, 3, 6 and 10 hours after S-phase, were quantified using ImageQuant software (the numbers are average band intensities (analyzed as volumes using the software and automatically corrected for local averages)) and graphed in **C**. Error bars represent the average of the two bands. Open bars in C represent 0 hour after S-phase, striped bars 0 to 3 hours, grey bars 3 to 6 and black chequered bars 6 to 10 hours after S-phase. Arrows showed the bands representing the loading control for each lane. The normalized background values were around 18% for clone 5 and 3% for clone 17.

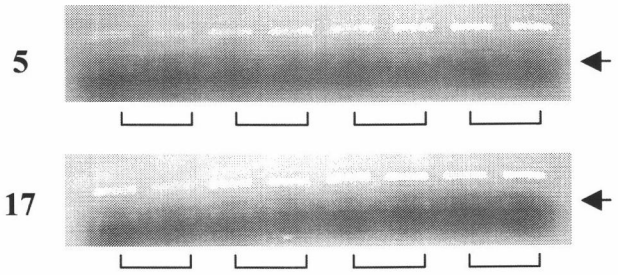
A Clone 5



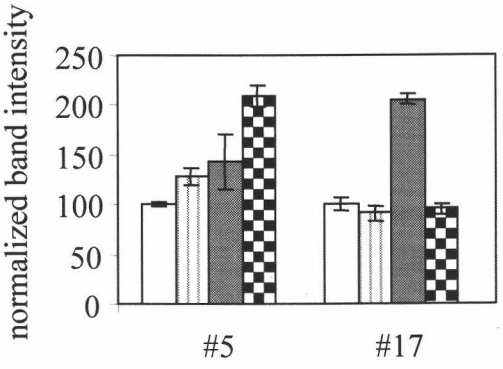
Clone 17



B



C



comparison between bands in each lane. Although inherent drawbacks of the assay exist, the result together with the previous observation from the FISH experiment can still be used to determine the replication timing of the *gfp-amb* gene in each clone.

From quantitative PCR measurements (Figure 4.6), clone number 5 seemed to have a gradual increase in band intensity with the highest peak at 6-10 hours after S-phase. The last peak (6-10 hours after onset of S-phase) did not decrease in intensity (as expected when replication of the gene occurred earlier than 6-10 hours after onset of S-phase); therefore, most of the replication of the gene probably did occur at late S-phase (6-10 hours). Earlier replication (gradual increase in band intensities earlier in S-phase) could be attributed to the somewhat lower degree of synchrony of the culture (~80% synchrony) and higher background reading. It could also be attributed to the *gfp-amb* replicated close to the 6-hour border in late S-phase. This result of the FISH experiment also showed that clone 5 was the later replicating clone of the *gfp-amb* gene out of the two clones tested which corresponded with the result of the quantitative PCR experiment (Figure 4.6).

Clone number17 had the expected two-fold increase in band intensity in the middle of S-phase (between 0-3 to 3-6 hours after onset of S-phase). The result of FISH experiment for clone 17 indicated that the *gfp-amb* gene was replicated earlier than clone 5. Taken together, these results suggested that clone 17 was a middle replicating clone of *gfp-amb* gene.

gfp-amb REVERSION RATE MEASUREMENTS

One can obtain measurements of spontaneous mutation rates without the complication of the fluctuation theory (Luria and Delbruck, 1943) if one can purge from the cell populations the mutants that had already arisen as a result of growing the population to high enough cell number (Bachl *et al.* 1999). When spontaneous mutation is measured in parallel cultures without selection against mutants during

growth, the standard deviation between the replicate cultures would be greater than the mean, and hence the need for fluctuation theory.

Using the *gfp-amb* gene product which does not exhibit a green fluorescence phenotype, one can sort and collect cells which have not reverted to the green fluorescence phenotype by using Fluorescence Activated Cell Sorting (FACS) after growing the cell population to high enough number to allow the detection of spontaneous mutagenesis in one generation time. In principle, one can then detect the linear increase in revertant frequencies of the *gfp-amb* gene of each clone, which would represent the spontaneous mutation rate of the gene in each clone (Bachl *et al.*, 1999).

Figure 4.7 shows a population of HeLa cells that had been transiently transfected with pEGFP-C1. The fluorescence intensity of GFP from pEGFP-C1 was readily distinguishable from the nonfluorescent HeLa cell background. Before sorting about 15% of the cells were fluorescent. Using the MoFlo high speed FACS machine, two-way sorting which resulted in enrichment of both populations were done. As shown, after the sort each population; *i.e.*, fluorescent and non-fluorescent HeLa cells are of 99% purity. The phenotype of cells from each population can be confirmed using a fluorescence microscope.

Using fluorescent HeLa cell populations as above, the flow cytometry instrument gating was set to define "a fluorescent HeLa cell". Revertant frequency is defined as the fraction of cell population that is fluorescent as defined by the instrument gating compared to total number of cells run through the flow cytometer.

Clones number 5 and 17 were analyzed repeatedly. In every case clone 5 accumulated more mutations than clone 17 after 5 days. The mutation frequency of clone 5 was higher than that of clone 17 in four experiments that was performed. However, there was considerable scatter on the mutation frequency data obtained daily for each clone. The small number of actual revertants collected in every experiment is a factor contributing to the scatter in the data (Table 4.2). Reversion of a single base pair in a single-copy gene is a very rare event; therefore, even though as

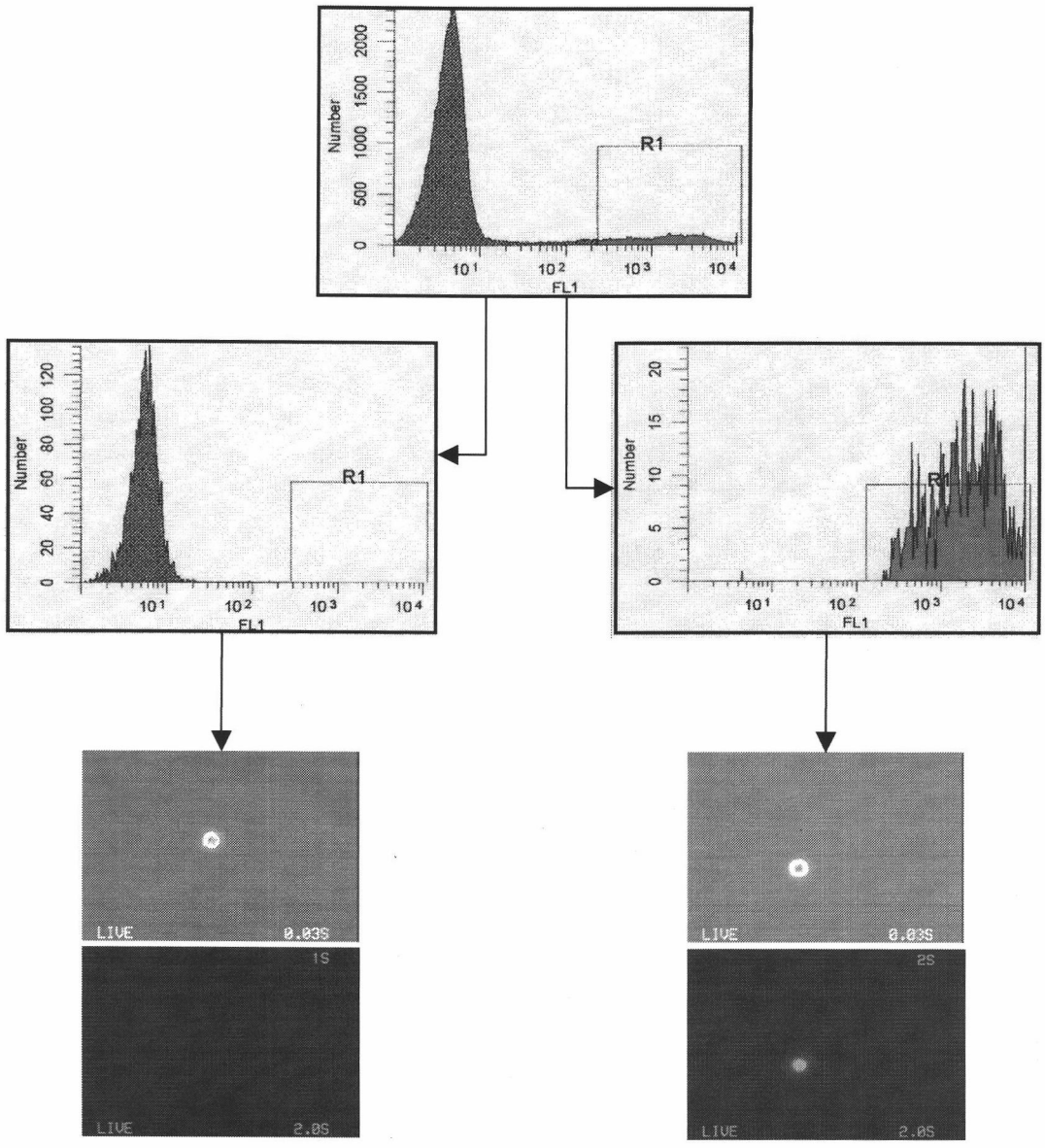


Figure 4.7. Determination of Fluorescence Gating for HeLa Cells Expressing GFP. PEGFP-transfected HeLa cells were used to determine the fluorescence intensity of GFP in HeLa cells. Two-way sorting using the predetermined gating resulted in populations of fluorescent (R1 gating) and non-fluorescent HeLa cells with more than 99% purity (enhancement of the population under R1 gating, *i.e.*: the right graph of cell number *versus* fluorescence intensity) . This gating (R1) was used in the flow cytometry analysis to determine whether a cell has reverted its *gfp-amb* gene to give a fluorescent phenotype of GFP (the top pictures of cells each represents a cell viewed using a light microscope and the bottom the same cell using fluorescence microscope).

many cells as possible were collected to run on the flow cytometry, the actual number of revertants collected was still small especially for the first day of the experiment. Nonetheless, the result of every paired experiment was consistent; therefore, it was concluded that in similar experimental conditions clone 5, the late replicating *gfp-amb* clone, accumulated mutants at least two fold more rapid than clone 17, the middle replicating *gfp-amb* clone.

Table 4.2

Comparison of Mutants Acquired in Clones 5 and 17

days	revertants accumulated			
	clone 5		*clone 17*	
	mutant	*total*	*mutant*	*total*
1	0	320,802	1	353,337
2	5	2,715,160	10	3,000,000
3	3	573,151	0	579,916
4	8	2,927,151	13	3,600,000
5	14	2,000,000	7	2,000,000

The spontaneous mutation rates expressed in number of revertants per cell per generation for both clones were derived from the experiment presented in Figure 4.8. The cells were doubling approximately every 36 hours, probably since they were trypsinized and replated repeatedly during the length of the experiment. The mutation rate for clone 5 was calculated to be about two per million cells per generation, whereas the mutation rate for clone 17 was calculated to be less than one per million cell per generation.

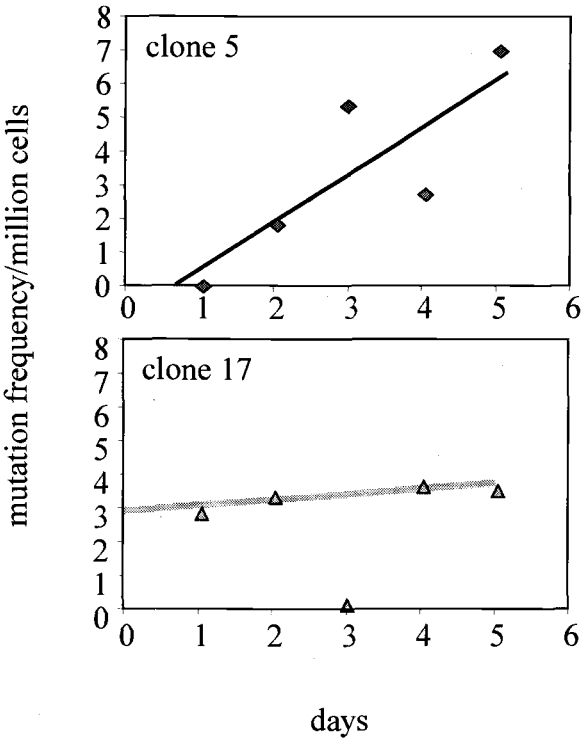


Figure 4.8. Estimation of Spontaneous Mutation Rates for Clones 5 and 17. Clone 5, which is the late replicating clone of *gfp-amb* gene, has a higher spontaneous mutation rate than clone 17, which was the middle-replicating clone. Clone 5 is represented in black diamonds and 17 in gray triangles. For clone 17, the revertant frequency at 3 days was not used in constructing the linear mutation rate curve.

DISCUSSION

The use of green fluorescent protein to study spontaneous mutation rates was put forth as an alternative to the complicated method of fluctuation analysis used to evaluate spontaneous mutation rate (Bachl *et al.*, 1999). These investigators used an amber mutant of *gfp* gene that is stably transfected in Δμ (a derivative of the A-MuLV-transformed pre-B cell line 18-81) cell line to evaluate the hypermutable rate

of these cells. In this report the use of the green fluorescent protein was adapted to determine whether the mutation frequency of a particular gene sequence varies depending upon whether the gene is replicated early or late in S-phase.

A tyrosine to amber mutation in the *gfp* gene sequence of the pEGFP-C1 was constructed, and HeLa cells were transfected with the linearized vector. Five clones that have the complete *gfp-amb* gene stably inserted into the chromosomes were isolated, and the replication timing of the *gfp-amb* gene in two of the clones was determined. For each clone, revertant frequencies of the *gfp-amb* gene which gave rise to green fluorescent HeLa cells were analyzed, and the mutation rates of the different clones were compared.

In adapting the technique of using *gfp* as the reporter gene for spontaneous mutation (Bachl *et al.*, 1999) in this study, there were several limitations. First, the expected spontaneous mutation rate in HeLa cells should be much lower than the rate of the hypermutable Δμ cells. Theoretically, a lot more than one million cells analyzed through the flow cytometer at one time was needed in order to detect just a few mutants. The sensitivity of the experiment would be higher a forward mutation, *i.e.,* analysing the number of non-fluorescent mutants from clones of HeLa cells stably expressing the green fluorescence protein (GFP) was done instead. However, it was difficult to isolate HeLa cell clones that were stably expressing the GFP. Most of the cells that were fluorescent after transfection with pEGFP would soon look unhealthy and rounded under the fluorescence microscope. After one month, none of the G418-resistant transfected HeLa cells were fluorescent, in contrast to ~20% at 48 hours after transfection. Whether the disappearance of the fluorescent HeLa cell population was due to cell death or slower doubling time of the fluorescent HeLa cells compared to the non-fluorescent cells was not determined. As mentioned before, Liu *et al.* (1999) have noted that expression of GFP seemed to be toxic in their mammalian cell lines and have linked the expression of GFP in these cells with the appearance of apoptotic markers. Their phenotypic observation of unhealthy cells

expressing GFP correlates well with my observation of the HeLa cells expressing the GFP.

Second, there seems to be a lag time for the fluorescence development of the GFP that was also observed by Bachl *et al.* (1999). The delay in the development of GFP fluorescence after expression in cells was noted previously (Kain *et al.*, 1995, Yang *et al.*, 1996, Tsien, 1998). At the time of sorting, some genes might already have a mutated DNA sequence but if the protein had been already expressed then it was not yet fluorescent. Without completely purging the cell populations of the mutant cells already present, each population would have a different number of revertants to start the experiment, and that could make estimating the spontaneous mutation rate inaccurate.

Some of the factors that complicated the analysis of mutation rate are intrinsic to the GFP, as was described above. The toxicity of GFP for HeLa cells possibly can lengthen the revertant cells' doubling time compared to the non-revertant cells or even killing the cells, which would greatly affect the observed number of revertants per day. Other factors are related to the experimental conditions, such as plating efficiency, cell growth density, the length that each cell population is subjected to sheath fluid in the sorting process, *etc.* Since HeLa cells used were not the HeLa S3 cells that can be adapted to spinner culture, for every time point the cell populations were trypsinized and a portion of the cell populations was used for the flow cytometry experiment. The rest of the cells were plated back with the old conditioned medium. And since the actual number of revertants was small, differences in plating efficiency can account for the observed scatter in the data. Another experimental condition that seems also to contribute to differences in observable mutation rate was cell density during growth. Boesen *et al.* (1994) noted a difference of 30-fold in observable mutation rate just by varying the cell density in cultures. To lessen the effect of experimental variations only comparison of mutation rates of clones in which data was taken from the same set of experiments were done.

For clone 5 and 17, repeated paired experiments suggested that there was a linear relationship between the observed revertant frequency and time for all of the cell populations tested. Clone 5, which was designated as a late-replicating *gfp-amb* clone, had always at least two-fold higher spontaneous mutation rate than did clone 17, which was a middle-replicating *gfp-amb* clone. In the paired experiments, clones 5 and 17 were affected similarly by certain experimental factors; therefore, the difference in the spontaneous mutation rate between the two clones should be valid. This observation indicates the existence of temporal differences in spontaneous mutation rates for human chromosomes. More studies would be needed to prove that our observation is a general phenomenon rather than an exception of the *gfp-amb* sequence used or the clones selected.

The implication that replication in middle S-phase is more accurate compared to late in S-phase is concurrent with slower replication rate (Collins, 1978; Collins *et al.*, 1980), lower average DNA accumulation rate and deoxyribonucleotide (dNTP) pools (Figure 4.9) in middle S-phase compared to late S-phase in HeLa cells. It is interesting especially since in the previous work it was shown that lowering the dNTP pool during DNA replication *in vitro* could result in lower mutation frequency observed (Chapter 2).

Is dNTP playing a role in controlling the S-phase DNA replication rate that in turn affects the spontaneous mutation frequency? It was also shown previously that dNTP pools in non-transformed fibroblast cell lines were lower than in HeLa cells (Chapter 2). If higher dNTP pools should concur with faster DNA replication rate, and dNTP pool levels in transformed cells are generally higher than in the non-transformed cells, then replication rates in transformed cell lines should be higher than in the non-transformed counterpart. This was indeed observed by Collins *et al.* (1980), who have shown that the S-phase DNA replication rate in WI-38 non-transformed cell line is slower in general compared to the 2RA cells, the transformed cell counterpart. Does the increase in replication rate of the transformed cell line also

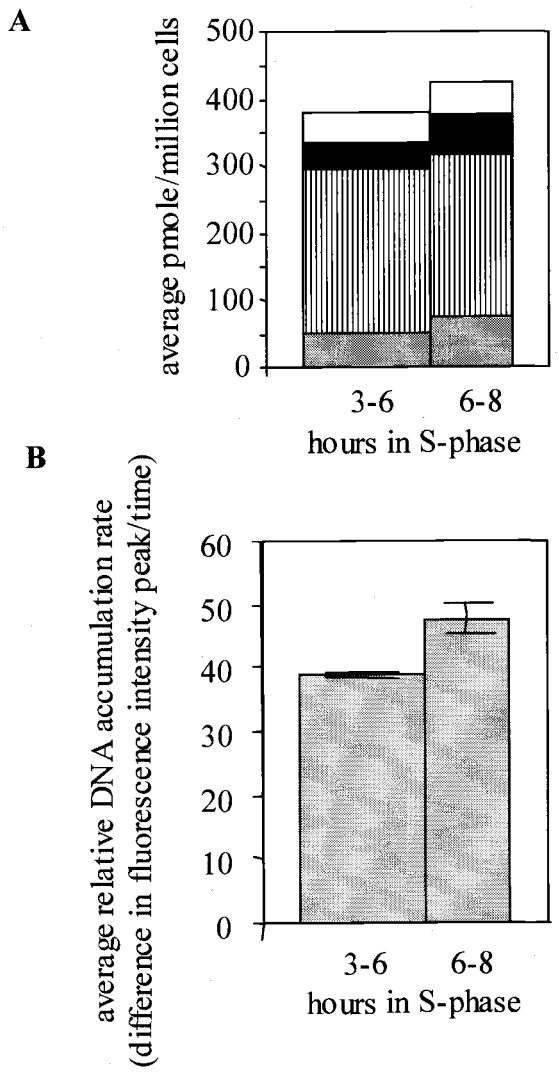


Figure 4.9. Average dNTP Pool Levels and DNA Accumulation Rates for Middle and Late S-Phase. This figure is adapted from data collected and graphed in Figure 3.8 in Chapter 3. **A** is the average dNTP pool levels and **B** is average DNA accumulation rates at middle and late S-phase. Grey bars in A represent dGTP, striped dCTP, white dATP and black bars dTTP.

increase the mutation rate in these cells? Clearly, the implication of the results presented here merits further experimentation.

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CHAPTER 5

DISCUSSION

Perturbation of dNTP pool concentrations in various prokaryotic as well as eukaryotic cells has long been documented to affect the observed mutation frequencies in these cells (reviewed in Meuth, 1989, Kunz and Kohalmi, 1991, Kunz *et al.*, 1994). However, in most instances the mechanistic relationships between alteration of the cells' "normal" dNTP concentrations and the resulting effects such as increases in substitution mutations or DNA strand breaks were not clear, and the proposed mechanisms of mutations involving dNTPs alteration do not explain the observable effects consistently. In the case of mammalian cells, to which the studies presented in this thesis are focused, the nature of the cells' chromosomal DNA replication that is not completely understood posed an added complication in understanding the relationship between dNTPs as building blocks for DNA replication and mammalian DNA replication fidelity.

The measured dNTP concentrations in cells during S-phase represent steady state dNTP concentrations resulting from the balance of dNTP production less the rate of their utilization in DNA replication and the rate of turnover reactions which maintain tight control over accumulation of dNTP pools in cells (Reichard, 1988). It was previously calculated that the measured dNTP concentrations in mammalian cells were only enough to support a few minutes of chromosomal DNA replication. This notion underscores the dynamic nature of the measured pools. From *in vitro* mammalian DNA replication studies employing SV 40 origin of replication, SV 40 Large T antigen and human cell extracts, it was noted that high dNTP pool concentrations during DNA replication increase mutation frequency and low dNTP pool concentrations during replication result in higher DNA replication fidelity (Kunkel, 1992a). High dNTP concentrations during replication are thought to result

in "next-nucleotide" effects (Kunkel, 1988), effectively "sealing-in" any mismatch in the previous template-DNA pair due to the enhanced rate of subsequent polymerization over a mismatch. With low dNTP concentrations during replication, it is thought that sufficient time for proofreading of a mismatch in the previous template-DNA pair is facilitated by slow DNA polymerization rate. However, kinetic mechanisms of DNA polymerization indicated that incorrect incorporation involves an additional rate-limiting step not observed for correct polymerization (reviewed in Johnson, 1993). The kinetic constants for exonucleolytic partitioning indicate that polymerization over a misincorporated nucleotide is extremely slow even without pool imbalance, questioning the significance of "slower" polymerization when next-nucleotide to be incorporated is low in concentration.

In mammalian cells *in vivo*, dNTPs are produced in cytoplasm while DNA replication occurs in the nuclei. There is strong evidence of the tight regulation of dNTP pools in cells (reviewed in Reichard, 1988). It has been noted also that replication fork movement in S-phase in HeLa cells increases as cells progress through S-phase. Nonetheless, the relationship between the measured dNTP pools and DNA replication rate in mammalian cells in S-phase has not been established. Another consideration for mammalian chromosomal DNA replication is the extremely large amounts of DNA that need to be replicated in short periods of time. It would seem that replication in a mammalian cell at less than an optimum polymerization rate is not an advantage.

In Chapter 2 of this thesis, the effect of the under-representation of dGTP in the measured mammalian cells' dNTP pools is studied using the *in vitro* SV 40 DNA replication system. Most of the studies involving observed increases in cells' mutation frequency that correspond to "imbalances" or alterations of the ratio of the four dNTP pools in the cells attributed the dNTP imbalance as the source of the increase in mutation. Higher concentration of one dNTP over others is thought to increase the possibility that the over-represented dNTP will be misincorporated more frequently since the nature of polymerization of a template with four different

possible substrates lends itself to substrate competition to the active site. However, as shown in Chapter 2, in the *in vitro* replication reaction, high concentration of one dNTP relative to other dNTPs did not seem to increase its misincorporation rate but increases the mutation frequency of the mismatch immediately before incorporation of the over-represented dNTP in DNA. Replication with biologically biased dNTP concentrations based on the calculated HeLa nuclear dNTP pools (60, 60, 30 and 10 μM for dATP, dTTP, dCTP and dGTP respectively, Leeds *et al.*, 1985) did not affect the observed mutation frequency when compared to replication with equimolar dNTP (100 μM) concentrations. It is possible that this *in vitro* observation is a consequence of the next-nucleotide effect. When dNTP concentrations are at certain ranges, lowering one dNTP pool did not immediately affect the observed mutation frequency since there is no considerable changes in the ability of other dNTPs to seal the resulting mismatches through next-nucleotide effect. To put it differently, when dNTP levels during replication are in the range of the K_Ms of mismatched extensions or lower, small changes in the dNTP ratio that may cause insertion errors are not affecting the mutation frequency since the concentration of the next correct nucleotide is not high enough too seal any errors made.

Very low mutation frequency is observed when replication is done with dNTPs at calculated diploid fibroblast concentrations. All dNTP levels in the diploid fibroblast concentrations were about three- to four-fold lower than biologically biased dNTP pool calculated from HeLa cells. Both HeLa and diploid fibroblast dNTP concentrations used in the *in vitro* replication reactions are significantly higher than the reported K_d for pol δ-dNTP binding (0.93 μM) or K_M for dNTP (0.067-1.2 μM depending on the presence or absence of PCNA) (Einolf and Guengerich, 2000). This should imply that in both reactions the polymerization rate should be maximal; however, a slight decrease in polymerization rate was also observed for reactions with diploid fibroblast concentrations. Even if some of the decrease in mutation frequency is due to slow polymerization to facilitate proofreading, the result of the decreased ability to seal-in most mismatches due to low concentrations of next

correct nucleotides available in replication reaction with lower dNTP concentrations should also play a factor. In the misincorporation kinetic study done for T7 DNA polymerase, the K_M for the correct dNTP addition over a mismatch is only four times higher than that of correct dNTP over correct dNTP (Wong *et al.*, 1990). Kinetic studies of DNA polymerase δ to date indicated similarities of polymerization kinetics between polymerase δ and other prokaryotic DNA polymerases that have been studied more extensively (Einolf and Guengerich, 2000). If the difference in K_Ms for correct addition *versus* incorrect addition over a mismatch observed for T7 DNA polymerase also holds true for mammalian DNA polymerases, K_M for mismatches that is only four-fold higher than K_M for correct polymerization would suggest that in the SV 40 *in vitro* replication reaction with biologically biased dNTP calculated for HeLa cell nuclei or with 100 μM equimolar dNTP concentrations, significantly higher numbers of mismatches can be sealed-in at least initially by mechanism of next-nucleotide effect when compared to reactions with diploid fibroblast dNTP concentrations. Of course *in vivo*, the sequence specific and other factors influencing replication fidelity in different regions in mammalian genome probably modulate the effect of dNTP levels on mutation frequency observed.

In chapters that followed, the relationship between overall level of dNTP in cells and the observed mutation frequency was studied. This notion is distinct from the widely observed increases in mutation frequency as the result of changes in the ratio of the four-dNTP pools in cells. The slow rate of mutation of the middle-replicating HeLa *GFP-amb* clone *versus* faster rate of mutation of the late-replicating clone (Chapter 4) corresponded with the modulation of the measured dNTP levels and DNA accumulation rates during S-phase in HeLa cells (Chapter 3). HeLa cell dNTP pool analysis during S-phase showed that the dNTP pool in the middle of S-phase is relatively low compared to dNTP pool levels in late S-phase. Concurrent analysis of DNA accumulation rate during S-phase showed that lower average total dNTP pools in middle S-phase corresponded to lower average DNA accumulation rate, although no conclusions can be drawn when more detailed study of dNTP pool

levels and DNA accumulation rates during S-phase were conducted. Although the actual replication fork growth in different time in S-phase was not measured in the study, several reports have shown that the rate in replication fork movement increases in HeLa cells as the cells proceed through S-phase (Painter and Schaeffer, 1971, Housman and Huberman, 1975). Chromosomal mammalian DNA replication has been shown to involve other DNA polymerases such as DNA pol ε, and *in vivo,* other factors can modulate DNA replication rate as well as dNTP pool levels in ways that are not clear to date. To substantiate the direct effect of dNTP pool levels on DNA replication rate, measurement of changes in the replication fork growth in response to experimentally introduced changes in dNTP pool levels at different time in S-phase is needed. Experiments measuring the change in replication fork movement in response to an introduced increase in dGTP level would provide convincing evidence that low dGTP pool levels affect DNA replication rate.

Higher dNTP concentrations in late S-phase in HeLa cells corresponds to the observation of higher mutation rate of the late-replicating GFP-amb HeLa clone, and lower overall dNTP concentrations in the middle of S-phase corresponds to a lower mutation rate of the middle-replicating clone. These observations agree with the results obtained from *in vitro* replication study. Although more studies need to be done, it is tempting to speculate that the increase in mutation rate seen is the result of the increase of dNTP pool levels during replication and also the replication rate.

Higher dNTP pool levels and hence replication rate can relate to the higher mutation rate observed. Kinetic studies suggest that the enhanced rate of binding of excess nucleotide to the enzyme-DNA complex does not necessarily result in "sealing-in" the mismatch directly 5' to it since the rate limiting step for polymerization of the mismatched nucleotide is the formation of the phosphodiester bond, which is extremely slow for a mispair. Moreover, it has been observed that the proofreading exonucease activity not only can excise mismatches present in the primer-template termini but it can also correct "buried" mismatches. An increase in the proportion of the enzyme-mismatch DNA complex that is bound to the next

correct nucleotide (as the result of higher dNTP levels during replication) probably does not increase the efficiency of mismatch extension. However, the [enzyme-mismatch DNA terminus-next correct nucleotide] complex can exist in a conformation that inhibits the movement of the primer-template DNA terminus into the exonuclease site and hence decreases the proofreading efficiency in the presence of high dNTP concentrations. Since the next two, three and four nucleotides after a mismatch are also present at high concentrations, the mismatch can effectively be "sealed-in" when all dNTPs are present at high concentration during replication. However, for *in vivo* DNA synthesis, other factors such as DNA mismatch repair, can still correct mispairs that are not corrected by the proofreading exonuclease during DNA replication.

It has been noted that in mammalian cells, most housekeeping and active genes are replicated early in S-phase while the non-transcribed genes replicate late in the S-phase. The indication that late-replicating genes acquires more mutations than middle-replicating genes seems to correspond well with the temporal regulation of genes. The average dNTP pool levels in mammalian cells also vary similarly during S-phase. The studies presented here indicate that dNTP levels during S-phase and hence DNA replication rate could play a factor in the modulation of DNA replication fidelity in mammalian cells.

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