Deoxyribonucleotides as Genetic and Metabolic Regulators


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Deoxyribonucleotides as Genetic and Metabolic Regulators

Christopher K. Mathews
Oregon State University
Department of Biochemistry and Biophysics
Corvallis, Oregon, USA

Correspondence
Department of Biochemistry and Biophysics
Oregon State University
2011 Agricultural & Life Sciences Bldg.
Corvallis, OR 97331-7305, USA

E-mail: mathewsc@onid.orst.edu

Running Title
dNTPs as Genetic and Metabolic Regulators
Nonstandard abbreviations: dNMP, dNDP, dNTP, deoxyribonucleoside mono-, di-, and triphosphate; rNMP, rNDP, rNTP, ribonucleoside mono-, di-, and triphosphate; RNR, ribonucleoside diphosphate reductase; MMR, mismatch repair
Abstract

For more than 35 years we have known that the accuracy of DNA replication is controlled in large part by the relative concentrations of the four canonical deoxyribonucleoside 5'-triphosphates (dNTPs) at the replisome. Since this field was last reviewed, about eight years ago, there has been increased understanding of the mutagenic pathways as they occur in living cells. At the same time, aspects of deoxyribonucleotide metabolism have been shown to be critically involved in processes as diverse as cell cycle control, protooncogene expression, cellular defense against HIV infection, replication rate control, telomere length control, and mitochondrial function. Evidence supports a relationship between dNTP pools and microsatellite repeat instability. Relationships between dNTP synthesis and breakdown in controlling steady-state pools have become better defined. In addition, new experimental approaches have allowed definitive analysis of mutational pathways induced by dNTP pool abnormalities, both in *E. coli* and in yeast. Finally, ribonucleoside triphosphate (rNTP) pools have been shown to be critical determinants of DNA replication fidelity. These developments are discussed in this review article.

Key words: mutagenesis, deoxyribonucleotides, nucleotide pools, oncogenes, cell cycle
It is now more than three decades since investigators first established that dNTP concentrations at replication sites are critical determinants of DNA replication fidelity and, hence, of spontaneous mutation rates. In recent years we have learned that maintaining nucleotide pools within controlled limits is relevant to additional cell processes, including cell cycle control, protooncogene function, mitochondrial function, defense against virus infection, DNA mismatch repair, and possibly telomere length determination and microsatellite repeat instability. I begin this article by considering how current technology has illuminated mutagenic pathways resulting from defined alterations in DNA precursor pools, and then move to other functions and processes. Figure 1 summarizes principal reactions in dNTP biosynthesis, identifying major allosteric control points.

MUTAGENIC MECHANISMS INFERRED FROM NUCLEOTIDE POOL VARIATIONS

Ribonucleotide reductase as the source of altered dNTP pools

It was perhaps inevitable that the earliest papers describing relationships between dNTP pool sizes and genomic stability in living cells involved altered allosteric regulation of ribonucleotide reductase, because of the function of this enzyme in synthesizing all four dNTPs (1). RNR is also at the center of more recent investigations, in which sequence analysis of mutant genes reveals defined mutagenic pathways in vivo resulting from specific pool imbalances (2,3). Kumar et al (2) studied the effects of three mutations in loop 2 of yeast RNR, a conserved region that connects the two allosteric control sites—the activity site and the specificity site—on the RNR large subunit, Rnr1. By correlating pool size abnormalities in each mutant with the mutation spectrum as revealed by sequence analysis in the CAN1 locus, conferring canavanine resistance, Kumar et al were able to establish consistently plausible mechanisms for each mutagenic event. Particularly important was their analysis of insertion and deletion mutations, in which slipped-strand intermediates, previously postulated from in vitro studies, rationalized the effects of specific pool imbalances on predicted misaligned pre-mutagenic intermediates. Mutagenic effects, which were large, overrode both proofreading and mismatch repair, both of which were active in these cells.

A different tack was taken in the *E. coli* system by Ahluwalia et al (3). These investigators used an unbiased approach to generate mutant forms of RNR that confer a mutator phenotype. Ahluwalia et al conducted random mutagenesis of a plasmid containing the *nrdA* and
nrdb genes, which encode the large (R1) and small (R2) proteins, each of which consists of two subunits. After transfection of the mutated plasmids into E. coli, a powerful selection technique was used to replace the resident nrdaB system with the mutated genes, and a papillation assay was devised to select for strains that showed elevated spontaneous mutagenesis. The 23 mutator mutations characterized clustered in three parts of the RNR structure: (1) the activity site, which responds to ATP and dATP; (2) the specificity site, which responds to dATP, dGTP, and dTTP; and (3) unexpectedly, a small region of the small (R2) subunit, which lies close to the activity site in the probable quaternary structure of the enzyme. All 23 mutator strains displayed dNTP pool abnormalities, which corresponded well with the mutational pathways that were stimulated. The mutants selected and the general approach should be of great value as investigators probe the structural basis for allosteric regulation in this central enzyme.

In fact, these strains have already proven to be useful for probing mechanisms both of mutagenesis and of regulation of RNR activity and specificity. Ahluwalia and Schaaper (4) studied one set of the specificity-site mutators, which showed dNTP pool alterations of about twofold, but enhancement of mutagenesis of 1000-fold or more—a phenomenon termed by the authors an “error catastrophe.” Evidence indicated that even these modest pool changes led to saturation of the mismatch repair activity. The MMR system appears particularly sensitive to changes in the dGTP pool, and that may explain the longstanding observations that dGTP is almost always the least abundant of the four canonical dNTPs.

**Mutagenesis involving enzymes other than ribonucleotide reductase**

*Nucleoside diphosphate kinase.* Mutations affecting nucleotide metabolic enzymes other than RNR have also proven instructive. In E. coli mutations affecting ndk, the structural gene for nucleoside diphosphate kinase, were shown some time ago to confer both nucleotide pool abnormalities and a mutator phenotype (5). NDP kinase converts the eight canonical rNDPs and dNDPs to the respective nucleoside triphosphates, so is involved in both DNA and RNA synthesis. The ndk mutations caused both a dCTP elevation and a dATP depletion; mechanisms for these effects are unknown. Nordman and Wright (6) argued that the dNTP pool alterations were not responsible for the mutator phenotype; their data suggested that the ndk mutant accumulated dUTP and that the mutator phenotype resulted from saturation of the DNA-uracil repair pathway. However, Schaaper and Mathews (7) pointed out the importance of using MMR-deficient strains in this type of analysis. When MMR-deficient strains were used in a lacZ
reversion assay (8), the ndk mutation predominantly stimulated A:T→T:A transversions. In the particular reporter system used, the next nucleotide was dCTP; hence, in the absence of MMR, even a modest dCTP elevation could provoke mutagenesis by a next-nucleotide effect.

**dCTP deaminase.** In the same study a dcd mutation, affecting dCTP deaminase, provoked transversion mutations by both G:C→T:A and A:T→T:A pathways (in *E. coli* the conversion of deoxycytidine nucleotides to deoxyuridine nucleotides occurs at the triphosphate level). The *dcd* mutation causes dCTP to accumulate. Again, as seen with the ndk mutants, sequence context was critical. For both reversions that were stimulated, dCTP, which accumulates in *dcd* mutant bacteria, was the next nucleotide.

Mutations in both ndk and dcd were used in a recent study by Gawel *et al* (9), who asked whether DNA replication fidelity in *E. coli* is higher on the leading or lagging strand. Even though in bacteria both strands are replicated by the same polymerase, previous studies had suggested that fidelity is higher for lagging-strand replication—a seemingly counterintuitive finding because of the more complex process of discontinuous DNA synthesis on the lagging strand. For reversions stimulated by the *ndk* or *dcd* mutations used in the previous study, the mutator effect will vary depending upon which strand undergoes the initial deoxyribonucleotide incorporation, because the next nucleotide in the template will be different in each case. Results of experiments in which reversion of each mutation was analyzed in both a leading- and a lagging-strand context supported the previous finding of greater lagging-strand replication accuracy.

**dCMP deaminase.** Deoxycytidylate deaminase is another enzyme that plays a critical role in controlling dNTP pool sizes. Through its allosteric activation by dTTP and inhibition by dCTP, the enzyme helps to keep in balance the pyrimidine dNTP pool. Early studies (1) suggested that mammalian cell mutants deficient in dCMP deaminase displayed a mutator phenotype resulting from dCTP accumulation and dTTP depletion. Kohalmi *et al* (10) showed that disruption of the gene encoding dCMP deaminase in budding yeast, *Saccharomyces cerevisiae*, showed the expected mutational specificity resulting from the pyrimidine dNTP imbalance. More recently Sánchez *et al* (11) showed that deletion of the dCMP deaminase gene in fission yeast, *Schizosaccharomyces pombe*, has a similar effect on dNTP pools but a more severe consequence in terms of genome instability, including replication fork collapse and activation of multiple DNA damage and checkpoint responses.
Another enzyme mentioned in this section is *E. coli* dGTP triphosphohydrolase. Another enzyme mentioned in this section is *E. coli* dGTP triphosphohydrolase, an enzyme of still unknown function that cleaves dGTP to deoxyguanosine and triplyphosphate, but evidently only in enteric bacteria. Gawel *et al* (12) made the serendipitous observation that insertional inactivation of *dgt*, the structural gene for this enzyme, conferred a mutator phenotype, with a specificity toward G:C ---\> C:G transversions and A:T ---\> G:C transitions. Both events could be stimulated by an increased dGTP pool, and that may well result from a deficiency of an enzyme that degrades dGTP. However, dNTP pools were not measured in this study; such data would be of great interest.

Polynucleotide phosphorylase. Intriguingly, Becket *et al* (13) reported that *E. coli* mutations that inactivate polynucleotide phosphorylase (PNP) confer an antimutator effect seen both for frameshift and substitution mutations. PNP catalyzes the phosphorolysis of RNA to rNDPs, which, of course, are the immediate precursors to deoxyribonucleotides via RNR. Whether the antimutagenic effect results from either dNTP pool changes or ribonucleotide depletion is a question of great interest. As discussed later in this article, ribonucleotide metabolism has recently been assigned a much larger role in DNA replication fidelity than previously suspected. On the other hand, dNTP pool changes may well be involved. Using an assay procedure that permits simultaneous determination of all four reactions catalyzed by ribonucleotide reducatase, our laboratory showed (14) that the activities of this enzyme are probably controlled by relative concentrations of the four rNDP substrates *in vivo*. Hence, a partial depletion of rNDP pools could decrease *in vivo* flux rates through RNR with a consequent depletion of dNTPs. As discussed elsewhere in this article, balanced dNTP depletions can enhance replication accuracy, by allowing for enhanced proofreading during DNA replication. In any event, it will be of great interest to learn the effects of polynucleotide phosphorylase mutations upon both ribonucleotide and deoxyribonucleotide pools.

**RIBONUCLEOTIDE METABOLISM AND DNA REPLICATION FIDELITY**

It has long been known that DNA polymerases will accept ribonucleoside triphosphates as substrates, albeit poorly, and that rNTP pools are much larger than dNTP pools in all cells examined to date. However, it is only recently that investigators have asked whether ribonucleotide incorporation into DNA in living cells might be a source of genomic instability. Nick McElhinny *et al* (15) examined the situation in yeast, where they found rNTP pools to
exceed the respective dNTP pools by factors of 36 to 190. Replicative DNA polymerases preferred dNTPs over rNTPs by several hundred- to several thousandfold, depending upon sequence context and the specific polymerase. The data suggested that one round of replication in vivo could result in 10,000 rNMPs incorporated into DNA, making ribonucleotide incorporation potentially the most abundant endogenous source of DNA damage. Data suggested (16, 17) that genomic instability resulted from strand misalignment during replication, due to the tendency of incorporated ribonucleotides to drive DNA from the B to the A conformation. rNTP incorporation has been shown also to slow the rate of DNA replication, due primarily to DNA polymerase stalling, particularly at sites where two or more consecutive ribonucleotides have been incorporated (18). In this latter study, which focused upon the E. coli replisome as well as yeast DNA polymerases, Yao et al estimated the frequency of ribonucleotide misincorporation at one in 2.3 kb during chromosome replication in vivo, a number similar to values reported for yeast replicative DNA polymerases (19). Studies with yeast replicative polymerases show varying abilities of polymerases to bypass an incorporated ribonucleotide or to excise it, depending upon sequence context, the specific polymerase, and the presence of more than one rNMP in tandem (19, 20, 21). These studies have recently been extended to human polymerase δ (22).

Several laboratories have shown that ribonuclease H2 plays a predominant role in initiating excision repair of sites where ribonucleotides have been incorporated (16, 22, 23, 24). These findings are of great interest for two reasons. First, a genetic deficiency of RNase H2 is associated with a rare hereditary neuroinflammatory disorder called Aicardi-Goutières syndrome, a finding that underscores the importance of this enzyme. Second, these findings have led to identification of the probable mismatch repair system in eukaryotes and in bacteria lacking methyl-directed mismatch repair. It has long been known that bacteria with the dam methylation system recognize unmethylated GATC sites to identify daughter DNA strands and correct mismatches accordingly. How might eukaryotes and prokaryotes lacking the dam system recognize the daughter strand? Several groups have presented evidence that the presence of non-excised ribonucleotides in DNA provides just such a marker. Lujan et al (25) have shown that inactivation of RNase H2 in yeast reduces MMR efficiency preferentially on leading-strand errors, suggesting that mismatch repair directed by RNase H2 is directed primarily toward leading-strand replication errors.
BALANCED dNTP POOL FLUCTUATIONS AS A FIDELITY DETERMINANT

The early interest in dNTP pools as fidelity determinants focused attention upon effects of unbalanced pools, caused, for example by mutations affecting RNR or dCMP deaminase, as discussed earlier in this article. An early indication that proportional accumulation of dNTPs might be mutagenic came when Chabes et al (27) mutated the yeast RNR activity site and found that dNTP pools expanded by 11- to 17-fold during DNA damaging treatment, with a disproportionate increase in mutation rates. They proposed that mutagenesis resulted from elevated dNTP concentrations in vivo, which permitted efficient translesion DNA synthesis.

A different, probably complementary, explanation came from work in our laboratory, not involving DNA damage as a fidelity determinant (28). In our study dNTP pools in E. coli expanded near-uniformly as a result of engineered RNR overexpression. Mutation frequencies increased out of proportion to the pool expansions. Because DNA polymerase-catalyzed chain extension from a mismatched 3’ terminal nucleotide has a much higher $K_M$ than does extension from a matched terminus (29), we proposed that as intracellular dNTP concentrations increase, the ratio of incorrect to correct chain extension increases. In essence polymerase-catalyzed proofreading is inhibited because the rate of mismatch extension increases at high [dNTP] with no significant effect upon the rate of extension from a correctly matched 3’ terminus. Since then at least two other studies, one in yeast (30) and the other in E. coli (31), have found elevated dNTP pools to be correlated with increased mutagenesis. In both studies the authors describe the pool expansion as a response to DNA damage; such responses include activation of ribonucleotide reductase. To the extent studied, it appears that uniform dNTP accumulation is mutagenic whether or not DNA damage is involved.

If proportional dNTP accumulation stimulates mutagenesis, what about dNTP depletion? Laureti et al (32) reported a correlation between reduction of dNTP pools and enhanced DNA replication fidelity. However, these investigators induced the dNTP depletion by disruption of energy metabolism resulting in ATP depletion, and they did not investigate other possible consequences of ATP depletion that might also have an effect on mutagenesis. Indeed, an opposite conclusion was reached by Bester et al (33), who inactivated genes that are normally up-regulated in carcinogenesis. This led to dNTP depletion accompanied by genomic instability.
Clearly, to the extent that mutagenesis is related to carcinogenesis, it is important to better understand the parameters that relate intracellular dNTP concentrations to mutagenesis.

**dNTP POOLS AND REPLICATION RATE CONTROL**

Are DNA replication rates subject to substrate-level control? A variety of studies suggest not. Average estimated intracellular dNTP concentrations are in the 5-to-35 µM range (reviewed in 34), and studies with synchronized cells show that dNTP pool sizes fluctuate dramatically during the cell cycle, with maximal values, as expected, during S phase. $K_M$ values for eukaryotic DNA polymerases are low; for example, Dresler et al (35) reported that DNA synthesis in permeabilized mammalian cells proceeded with $K_M$ values in the 1- to 3-µM range. These and other data suggested that average dNTP concentrations within eukaryotic cell nuclei are severalfold higher than polymerase $K_M$ values, and hence, that replisomes operate at substrate saturation or close to it. The situation is different in prokaryotic systems (36), but it appears that their replisomes also function at $V_{max}$.

The question whether eukaryotic replication proceeds at $V_{max}$ with respect to dNTP concentration has received critical attention only recently. Poli et al (37) increased intracellular dNTP pools in budding yeast, either by overexpressing the gene for the RNR R1 protein or by blocking expression of the RNR-inhibitory protein Mec1. In both cases the rate of DNA chain elongation, as measured by a DNA-combing assay, was increased, implying that, at least in this organism, dNTP pools are rate-limiting for DNA replication. However, the relationship is complex, at least in yeast. Chabes and Stillman (38) found that greater increases in dNTP pools, 10- to 35-fold, also brought about by stimulating RNR expression, overrode the DNA damage checkpoint and inhibited cell cycle progression. The relationships between dNTP metabolism, DNA damage, and cell cycle progression comprise a broad field that lies beyond the scope of this article. The question whether dNTP concentrations during S phase are rate-limiting for DNA replication merits critical attention.

**REGULATION OF dNTP POOLS BY CONTROLLED NUCLEOTIDE DEGRADATION**

Most of our early understanding of regulation of metabolite pool sizes focused upon control of biosynthetic reactions, partly because anabolic regulation appeared to make metabolic sense and partly because the earliest-investigated allosteric enzymes catalyzed biosynthetic
reactions. In deoxyribonucleotide metabolism the mechanistic and regulatory complexity of ribonucleotide reductase focused attention upon dNTP synthesis as the primary site of regulatory mechanisms. Still, several lines of evidence, primarily in Peter Reichard's laboratory, pointed to dNTP degradation as an important site for regulating dNTP pools (see 39 for a review of early evidence and 40 for a more recent review). Reichard and colleagues proposed that dNTP pools are controlled by substrate cycles, the interconversion of deoxyribonucleoside mono- and triphosphates. The problem in solidifying this reasonable concept was identifying specific enzymes in dNTP degradation and describing how their activities are regulated, and how this regulation might contribute to controlling dNTP pools.

The regulatory importance of dNTP breakdown became much clearer in 2011 with the simultaneous discovery in two laboratories of an enzyme, SAMHD1, that regulates dNTP pools by selectively degrading them to the respective deoxyribonucleoside plus triphosphate (41, 42). This protein (sterile alpha motif and HD domain-containing protein 1) was identified as a protein that restricts lentivirus replication in some myeloid cells and also as a component of the innate immune response. Moreover, genetic deficiency of this protein in humans was known to be associated with a form of Aicardi-Goutières syndrome, previously mentioned in this article as a consequence of RNase H2 deficiency.

Powell et al (41) and Goldstone et al (42) both reported that SAMDH1 is a dNTP triphosphohydrolase, comparable to the bacterial dGTP triphosphohydrolase mentioned earlier in this article, which cleaves dNTPs to the corresponding deoxyribonucleoside plus triphosphate. Both laboratories reported that activity of the enzyme is stimulated by dGTP, raising the interesting situation that the same molecule, dGTP, is both a substrate and an allosteric modifier of the enzyme. However, the enzyme is also stimulated by ribo-GTP, which is present in much higher concentrations, a factor that led Amie et al (43) to propose that GTP is the true allosteric activator. This latter group also showed that downregulation of SAMDH1 decreased sensitivity of HIV-1-infected cells in macrophages but not in T-cells, by increasing dNTP levels (44), and also they identified the end-gap repair process in viral integration as the sensitive step in HIV-1-infected human macrophages (45).

The dNTPase activity explained how SAMDH1 could restrict lentivirus growth—simply by depleting dNTP pools below the level required for viral reverse transcription (45). Some
viruses, notably HIV-2, fight back. These viruses encode a protein, Vpx, which targets SAMHD1 for degradation and prevents it from depleting dNTP pools (41).

Franzolin et al (46) have shown that the enzyme plays a broader metabolic role than simply guarding blood cells against virus infection. These investigators found the enzyme to be widely distributed in mammalian cells and tissues. Treatment of cells with short inhibitory RNAs directed at SAMDH1 caused dNTP pools to expand. Variable expression of SAMHD1 during the mammalian cell cycle supported the idea that the enzyme helps to keep dNTP levels low during G1. Hence, these findings indicate that SAMDH1 contributes to the control of dNTP levels in most cells, possibly to the same extent as ribonucleotide reductase.

The SAMDH1 protein was originally described as a homodimer (42), but subsequent investigation has revealed that inactive monomeric and dimeric forms of the protein tetramerize in the presence of dGTP (47, 48, 49). Ji et al (48) showed that two molecules of dGTP bind at each allosteric site. dGTP binding and tetramerization force a conformational change that facilitates binding of all dNTP substrates at the catalytic site.

An interesting comparison has been drawn to bacterial dNTP triphosphohydrolases, particularly the enzyme from Enterococcus faecalis, with which the human enzyme shares 36 percent sequence identity (50). The bacterial enzyme is a homotetramer, even in the absence of effectors. It contains two different classes of binding sites. dGTP binding at the primary site apparently acts as an “on-off switch,” while the secondary site can bind dNTPs that serve either as activators or inhibitors of the hydrolysis of specific dNTPs at the catalytic site. The interesting question how this system, comparable in complexity to rNDP reductase, acts to regulate dNTP pools in these bacteria, and why, remains to be explored.

An SAMDH1-independent pathway for counteracting HIV-1 infection has recently been described (51). Thus pathway acts at the level of dNTP synthesis, not breakdown. In this study the cyclin-dependent kinase inhibitor p21 was found to down-regulate expression of the RNR R2 protein, by repressing the E2F1 transcription factor, thereby inhibiting transcription of the RNR2 gene.

A recent paper (52) describes another protein, DCTPP1, which is suggested to be involved in controlling dNTP pools. This enzyme is described as a dCTPase, cleaving dCTP to dCMP and pyrophosphate. Knockout of this enzyme in human cells caused pyrimidine dNTP
pools to expand. Because data on dATP and dGTP pools were not included in the study, the function of this enzyme in regulating dNTP pools is not clear.

OXIDIZED dNTPs AND GENOMIC STABILITY

In 1992 the finding (53) that the mutT gene product of E. coli is a nucleoside triphosphatase focused intense interest upon damaged nucleotides as premutagenic lesions. It is well known that the major mutagenic target of reactive oxygen species is guanine, which in DNA undergoes oxidation to 8-oxoguanine. Properties of the E. coli MutT protein and its human homolog, hMTH-1, suggested that guanine oxidation occurs at the nucleotide level, and that enzymatic hydrolysis of the oxidized nucleotide, 8-oxodGTP, “cleanses” the DNA precursor pool by eliminating a damaged nucleotide whose incorporation would be mutagenic.

These early findings stimulated an outpouring of papers supporting this reasonable conclusion and identifying other modified dNTPs that could similarly represent premutagenic lesions (reviewed in 54). Most of the approaches used, while persuasive, were indirect, in that they did not involve actual measurements of intracellular pools of damaged nucleotides—a difficult task because of their expected low pool sizes. In 2002 our laboratory was frustrated by our inability to detect 8-oxodGTP accumulation in mutT mutants of E. coli (55); in that study 8-oxo-dGTP could have been detected at a minimum intracellular concentration of 0.3 µM.

However, several years later, and with the use of a more sensitive detector, we were able to find and quantitate 8-oxo-dGTP in extracts of mitochondria from several tissues of the rat. We looked in mitochondria because of the highly oxidizing conditions that exist in this organelle. In collaboration with Thomas Kunkel's laboratory (56) we found that the estimated molar 8-oxo-dGTP concentrations within mitochondria were sufficient to have strong mutagenic effects, as determined by use of an in vitro replication error detection system. And that led us to wonder about a puzzling finding from our laboratory (57). Although, as noted earlier, dGTP is almost invariably the least abundant component of the dNTP pool, typically representing five mole percent or less of total dNTP, that ratio is reversed in extracts of rat tissue mitochondria, where dGTP may represent 90 percent or more of total dNTP (58; also see Table I). Although we and others have not seen such profound dNTP asymmetry in cultured cells (59), and others have not detected dGTP excess in mouse mitochondria (60), the high dGTP pool in rat tissue mitochondria is a consistent finding in our laboratory.
What purpose might this dGTP accumulation serve? One speculative explanation is that it serves to “buffer” the biological consequences of dGTP oxidation, by mixing 8-oxodGTP with the much larger dGTP pool and reducing proportionately the incorporation of the damaged nucleotide into DNA. Readers of this article who can think of straightforward experiments to test this hypothesis are encouraged to contact the author.

dNTP METABOLISM, CELL CYCLE CONTROL, AND ONCOGENES

There is a growing body of literature describing interrelationships between dNTP pool regulation and the action of protooncogenes and cell cycle regulators. This is a challenging area to review, partly because of work done in yeast and mammalian cells, which have similarities but also distinctions in their modes of cell cycle regulation, partly because the biochemical actions of some of the protooncogenes are not yet known, and partly because it is not always clear whether changes in dNTP pools are causes or effects of regulatory phenomena. What follows is somewhat disconnected and incomplete, offered primarily to give the reader entré to some of the relevant literature.

Like multicellular organisms, the budding yeast *Saccharomyces cerevisiae* has an elaborate genome integrity checkpoint, which senses replication stress that might lead to DNA damage and acts to relieve that stress. Part of the checkpoint response acts, via the Mec1-Rad53-Dun1 pathway, upon ribonucleotide reductase, to ensure that adequate and balanced dNTP pools are available (61). The human homologs of Mec1 and Rad53 are upstream regulators of p53. Dun1 is a protein kinase that acts, either during normal S phase or following DNA damage, to phosphorylate Smi1, a protein inhibitor of RNR activity, targeting it for proteolysis, and thereby relieving that inhibition and stimulating dNTP synthesis (62). Phosphorylation of another protein, Dif1, causes release of two RNR small subunits, Rnr2 and Rnr4, from the nucleus to the cytoplasm, where they combine with Rnr1, a large subunit, to form an active holoenzyme. Full activation of the Mec1-Rad53-Dun1 checkpoint after DNA damage causes the dNTP pools to increase by 6- to 8-fold in mostly balanced fashion. An additional gene, *Ixr1*, interacts with *dun1*, to promote the expression of *RNRI* and full activation of ribonucleotide reductase (63).

These findings are somewhat reminiscent of an earlier report (64) that unbalanced nucleotide pools in human cells, arising as a consequence of DNA damage, triggered p53 activation through the human counterpart to the Mec1-Rad53-Dun1 pathway. However, in that
study, the pool imbalance was induced by \( N \)-\((\text{phosphonoacetyl})\)-L-aspartate, an inhibitor of pyrimidine ribonucleotide biosynthesis. Hence, it was not possible to ascribe the results specifically to abnormalities in deoxyribonucleotide pools.

As discussed earlier, abnormal dNTP pool ratios are highly mutagenic. To what extent does the genome integrity checkpoint act to recognize unbalanced pools and minimize mutagenesis? To answer this question Kumar et al (65) generated several RNR loop 2 mutations affecting the specificity site, as mentioned earlier in this article (2). These mutant strains had elevated mutagenic potential. The checkpoint responded when one or more dNTPs were depleted, but not when all four pools were adequate, even if unbalanced. Hence, the consequences of highly unbalanced pools per se are not sensed as sufficiently damaging to activate the checkpoint. More recent analysis (66) shows that pre-activation of the genome integrity checkpoint increases tolerance to multiple DNA-damaging agents, a finding that helps explain the development of resistance, in humans, to certain chemotherapeutic agents.

Our laboratory has been involved in a collaboration with M. Nikiforov on control exerted by the C-MYC oncogene on dNTP metabolism (67). Down-regulation of C-MYC caused specific repression of the genes for thymidylate synthase, inosinate dehydrogenase 2, and PRPP synthetase 2, with corresponding depletion of dNTP pools and cell cycle retardation. Overexpression of C-MYC had the opposite effects: enhanced expression of the three target genes and elevation of the dNTP pools. The data suggested that a major action of C-MYC is regulation of dNTP synthesis, exerted by control of the expression of the three enzymes just mentioned.

More recently we described evidence supporting a role for dNTP metabolism in oncogene-induced senescence (68,69). Human fibroblasts undergoing senescence induced by \( \text{HRAS}^{\text{G12V}} \) expression underexpressed both ribonucleotide reductase and thymidylate synthase, and possessed subnormal dNTP pools. Reciprocally, ectopic coexpression of the two enzymes, or addition of exogenous deoxyribonucleotides, suppressed DNA damage and senescence-associated phenotypes.

Another collaboration, between our laboratory and that of K. Huebner (70) suggested a more specific relationship between thymidine nucleotide metabolism and expression of the \( \text{FHIT} \) oncogene. An early genetic alteration in preneoplasia is alteration of chromosome fragile sites, leading to loss of Fhit protein expression. In several cell lines we found DNA defects
accompanying loss of Fhit expression that could be traced to decreased expression of thymidine kinase 1, with a consequent specific decrease in dTTP pools. The effects could be reversed or prevented by addition of sufficient exogenous thymidine.

Finally, a relationship has recently been described between RNR levels and expression of the antiapoptotic protein Bc12 (71), which mediates inhibitory effects on the G1-to-S-phase transition. Overexpression of Bc12 in transgenic mice promotes development of lymphomas. The study by Xie et al (71) showed that expression of Bc12 exerts its effects partly by decreasing RNR activity, with consequent depletion of dNTP pools. At least part of this effect is brought about by disrupting the interaction between RNR R1 and R2 proteins, necessary to form a functional holoenzyme.

TELOMERES AND MICROSATELLITE REPEATS

I conclude this article by briefly mentioning two recent papers highlighting intriguing relationships between dNTP metabolism and other significant cellular processes. Gupta et al (72) pointed out that telomeric DNA sequences are guanine-rich, while dGTP is, as noted earlier, the least abundant dNTP. Examining telomere lengths in yeast, these investigators found relatively modest effects upon telomere length from varying dNTP pool sizes. However, varying the ratios of dNTPs had major effects. In particular, telomere length was most sensitive to changes in the relative dGTP concentration. This effect occurred primarily at the level not of rate, but of processivity. These findings led Gupta et al to suggest that telomerase activity is modulated in vivo by dGTP levels. The relationships between telomere metabolism and cancer and other disease states may focus increased attention on the biochemical reasons why the dGTP pool is so low in most cells.

The other study, primarily a speculation at this stage, pertains to dNTP metabolism and the generation of trinucleotide repeats (73). It is generally accepted that repeats are generated by template-primer slippage during DNA replication. Kuzminov (73) speculated, for example, that replication of a C-rich template could cause localized depletion of dGTP in the vicinity of a replication fork, a condition that could retard replication, favoring slippage. The question here, of course, is the rate at which locally depleted dNTP pools can be refilled by inward diffusion to replication sites. Issues related to rates of intracellular diffusion of metabolites within cells have
been of considerable interest in the past (74). Experiments to examine localized pool depletion with respect to formation of triplet repeats should be of considerable interest.

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REFERENCES


repression restricts HIV-1 replication in macrophages by inhibiting dNTP biosynthesis pathway. 

*Proc. Natl. Acad. Sci. USA* E3997–E4006


FIGURE LEGEND

Figure 1. Pathways of de novo dNTP biosynthesis in mammalian cells. Salvage pathways are not shown. Each enzyme name is in italics. Allosteric effectors are shown with upward and downward arrows, denoting activators and inhibitors, respectively.

TABLE I

Estimated dNTP Concentrations in Mitochondria from Rat Tissues. Data are from reference 56.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Estimated Intramitochondrial Concentration µM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dATP</td>
</tr>
<tr>
<td>Liver</td>
<td>1.7</td>
</tr>
<tr>
<td>Heart</td>
<td>2.1</td>
</tr>
<tr>
<td>Brain</td>
<td>3.5</td>
</tr>
<tr>
<td>Skeletal muscle</td>
<td>1.6</td>
</tr>
<tr>
<td>Kidney</td>
<td>2.4</td>
</tr>
</tbody>
</table>
Figure 1