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Mutational consequences of dNTP pool imbalances in *E. coli*

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ABSTRACT

The accuracy of DNA synthesis depends on the accuracy of the polymerase as well as the quality and concentration(s) of the available 5′-deoxynucleoside-triphosphate DNA precursors (dNTPs). The relationships between dNTPs and error rates have been studied *in vitro*, but only limited insights exist into these correlations during *in vivo* replication. We have investigated this issue in the bacterium *Escherichia coli* by analyzing the mutational properties of *dcd* and *ndk* strains. These strains, defective in dCTP deaminase and nucleoside diphosphate kinase, respectively, are characterized by both disturbances of dNTP pools and a mutator phenotype. *ndk* strains have been studied before, but were included in this study, as controversies exist regarding the source of its mutator phenotype. We show that *dcd* strains suffer from increased intracellular levels of dCTP (4-fold) and reduced levels of dGTP (2-fold), while displaying, as measured using a set of *lacZ* reversion markers in a mismatch-repair defective (*mutL*) background, a strong mutator effect for G·C→T·A and A·T→T·A transversions (27- and 42-fold enhancement, respectively). In contrast, *ndk* strains possess a lowered dATP level (4-fold) and modestly enhanced dCTP level (2-fold), while its mutator effect is specific for just the A·T→T·A transversions. The two strains also display differential mutability for rifampicin-resistant mutants. Overall, our analysis reveals for both strains a satisfactory correlation between dNTP pool alterations and the replication error rates, and also suggests that a minimal explanation for the *ndk* mutator does not require assumptions beyond the predicted effect of the dNTP pools.

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1. Introduction

Cells employ multiple mechanisms to ensure an appropriate low error rate during the replication of their genome [1–3]. The responsible DNA polymerases (replicases) avoid errors through a combination of error prevention at the insertion step (base selection) and error correction *via* the exonucleolytic removal of erroneous insertions (proofreading or editing). Following polymerization, postreplicative mismatch repair (MMR) is capable of further reducing the error rate by detecting and correcting mispairs that escaped the proofreading step. In addition, cells employ numerous DNA surveillance and repair pathways aimed at maintaining the DNA in a damage-free state, so as to minimize the entry of damaged, and likely mutagenic, DNA into the replication fork [4].

One important additional factor that is relevant to DNA replication fidelity and has been receiving increasing interest is the status of the DNA precursors, the 5′-deoxynucleoside-triphosphates (dNTPs), both qualitatively and quantitatively. Quality-wise, the cellular dNTPs are 'sanitized' to rid the precursor pool of modified or damaged dNTPs that upon incorporation into the DNA may be toxic and/or mutagenic [5,6]. One example of such sanitizing activity is the MutT protein that removes 8-oxodGTP from the pools [5,7].

In terms of quantities, each of the dNTPs is kept at relatively low concentration in the micromolar range (at only a few % of the corresponding rNTPs). The reason why the dNTPs are kept low is an interesting question that may well relate to the need to keep the error rate low. In addition to overall low levels, the relative ratios of the four dNTPs (dATP, dTTP, dCTP, dGTP) are kept under control, although not at equimolar levels. In general the dGTP level is the lowest in both prokaryotes and eukaryotes [8,9] with the exception of mitochondria [10,11].

The regulation of the cellular dNTP pool occurs at a number of levels. One critical step involves allosteric regulation of the enzyme ribonucleotide reductase (RNR), which performs the important reduction of the ribonucleotides to the corresponding deoxyribonucleotides in an allosterically controlled fashion that (i) limits overall dNTP production and (ii) balances the reduction of the four substrates such that the resulting dNTPs are present in the cell at the desired proportions [3]. In both *Escherichia coli* and human cells this reduction occurs at the level of the nucleoside diphosphates (NDP→dNDP). Once formed, the dNDPs are converted to the dNTP level by the enzyme nucleoside diphosphate kinase (NDK).

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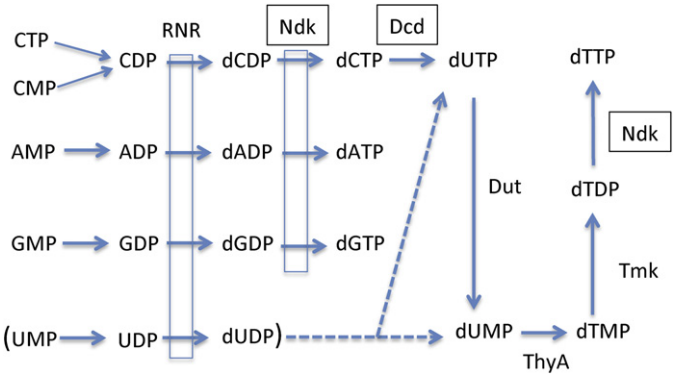

Fig. 1. Pathways for dNTP biosynthesis in *E. coli*. RNR = ribonucleotide reductase, Ndk = nucleoside diphosphate kinase, Dcd = dCTP deaminase, Dut = dUTPase, ThyA = thymidylate synthase, Tmk = dTMP kinase. The pathway in parentheses along with the stippled paths indicates the minor pathway (~20%) for dUMP (and dTTP) synthesis. The major pathway for dTTP synthesis is *via* dCTP and dCTP deaminase.

RNR is a unique enzyme containing two allosteric regulatory sites. The first one regulates overall activity through an (ATP/dATP-controlled) on/off switch (this site is termed the activity site). The second allosteric site determines which substrate can be reduced at any given time (called the specificity site) [3]. Recent studies have described strains of *E. coli* [12] and *S. cerevisiae* [13] carrying amino acid substitutions at sites of RNR allosteric regulation and, consequently, displaying a mutator phenotype (increased mutation rates). Likewise, overproduction of RNR itself is mutagenic [8,14], further supporting the important role of RNR in controlling the cellular mutation rate.

While RNR is a critical component in the dNTP metabolism, it is embedded within a larger network of nucleotide metabolizing pathways, the components of which may also affect the dNTP pools. We have outlined part of this network in Fig. 1. Of keen interest to our present study into the role of dNTPs and mutagenesis are two genes: *dcd*, encoding dCTP deaminase (Dcd) [15], and *ndk*, encoding nucleoside diphosphate kinase (Ndk) [16,17]. As shown in Fig. 1, dCTP deaminase is responsible for converting dCTP to dUTP, representing the first step in the *de novo* synthesis of dTTP [15]. dUTP is rapidly converted to dUMP by dUTPase (*dut* gene product); dUMP is converted to dTMP by thymidylate synthase, and dTMP is converted by two consecutive kinase reactions to the final product dTTP. Altered dNTP pools in *E. coli dcd* mutants lacking dCTP deaminase activity were reported early on [18,19]. As expected, these strains accumulate dCTP and, when grown on synthetic medium, suffer from reduced dTTP levels [18,19]. However, no studies on the mutation rates of *dcd* mutants have been reported, and therefore this strain was chosen presently to analyze relationships between dNTP pools and mutagenesis.

The second genetic defect that we chose for an in-depth analysis of mutagenesis and dNTP pools is *ndk*. Ndk is a multipurpose enzyme capable of converting each of the (d)NDPs to the corresponding triphosphates [20]. Interestingly, in *E. coli* the enzyme is dispensable as its function can be substituted, at least in part, by other kinases, particularly adenylate kinase [21,22]. Deletion of *ndk* caused both dNTP pool changes and a mutator phenotype, which was ascribed to the dNTP pool changes [22,23]. However, this particular explanation for the *ndk* mutator effect has been controversial [24,25]. One other study showed that complementation of the *E. coli ndk* defect with the human NDK homolog hNm23-H2 abolished the mutator phenotype but did not restore the dNTPs to their normal levels [24]. Hence, the dNTP pool alteration was not considered the main cause of the mutator phenotype. Instead, an alternative explanation was forwarded in which the main cause of the mutator effect is related to an increased production of dUTP and its incorporation into DNA instead of dTTP [24,25]. As noted (Fig. 1), dUTP resulting from the deamination of dCTP, is hydrolyzed by dUTPase (*dut* gene product) to yield dUMP, which is then converted to dTMP and eventually dTTP. As dUTP is a close analog of dTTP, differing only in the lack of the 5-methyl group, it is readily incorporated by DNA polymerase, and rapid destruction of dUTP is therefore necessary to prevent frequent incorporation. In additional support for the dUTP hypothesis, a synergistic interaction was reported between *ndk* and the *dut-1* defect, resulting in further enhancement of mutagenesis [24]. On the other hand, the intrinsic mutagenicity of uracil in DNA is low, as is the case for the abasic sites that result from its removal by uracil DNA glycosylase. Therefore, the precise mode of mutagenesis in *ndk* strains remains open to interpretation and is addressed by this study.

In this paper, we investigate in some detail the mutational properties of the *dcd* and *ndk* strains, focusing on the precise types of mutations that are enhanced (mutational specificity). In parallel, we determine the dNTP pool changes in the strains, and subsequently attempt to make meaningful comparisons between the pool changes and the observed mutational specificity. The mutation measurements are made in mismatch-repair-deficient strains (*mutL*) in order to more accurately correlate the dNTP data with replication error rates. Overall, the data indicate that meaningful correlations between dNTP pool changes and the induced replication errors can be derived.

2. Materials and methods

2.1. Strains and media

The *E. coli* strains used are derivatives of strain KA796 (Δ*pro-lac*, *ara*, *thi*) [26]. Strain constructions by P1 transduction were done using P1*virA*. Two mismatch-repair-deficient (*mutL*) derivatives of KA796 were used: NR9559 (*mutL211*::Tn*5*) [27] and NR12443 (*mutL218*::Tn*10*). The *mutL* markers in the two strains derive, respectively, from strains ES1301 and ES1481 [28]. The *dcd-12*::Tn*10dkan* allele [15] was introduced into KA796 and NR9559 (*mutL*) from BW1040 [15] (provided by Dr. Bernard Weiss, University of Michigan) yielding, respectively, NR12572 (*dcd*) and NR12573 (*mutL dcd*). The Δ*ndk*::*cam* allele was introduced into KA796 and NR12443 (*mutL*) from QL7623 [22] yielding, respectively, NR11814 (*ndk*) and NR11816 (*mutL ndk*). Into each of these strains we introduced, by conjugation, the series of F'*prolacZ* episomes originally present in strains CC101 through CC106 [29].

Liquid LB and minimal media (MM) were prepared using standard recipes [30]. Solid medium contained 1.5% agar (Difco). MM medium contained 1× Vogel-Bonner salts [31], 0.4% glucose (MM-Glu) or 0.4% lactose (MM-Lac) as a carbon source, and 5 μg/ml of thiamine. For experiments with *dcd* strains the solid media (LB, MM-Glu, MM-Lac) contained additionally 50 μg/ml of thymidine to improve colony growth on the plates (larger colony sizes). Liquid media, used for generation of mutant frequencies and extraction of cellular dNTP pools (see below), did not contain any added thymidine. Antibiotics, when required during strain constructions, were added at 25 μg/ml (kanamycin), 12.5 μg/ml (tetracycline), or 20 μg/ml (chloramphenicol); LB-Rif plates used for the scoring of rifampicin-resistant mutants contained 100 μg/ml rifampicin (Sigma–Aldrich).

2.2. Mutant frequency determinations

Mismatch-repair-deficient (*mutL*) strains were used for these series of experiments. For each strain, a total of 12–20 independent LB cultures (1 ml each) were initiated from single colonies (one colony per tube). The cultures were grown to saturation on a rotator

wheel at 37 °C. The total cell count in the cultures was determined by plating 0.1 ml of a 10^{-6} dilution on MM-Glu and LB plates. The number of *lac* revertants in each culture was determined by plating 0.1 ml of the undiluted cultures on MM-Lac plates. The number of rifampicin-resistant colonies in each culture was determined by plating 0.1 ml undiluted cultures on LB-Rif plates. The plates were incubated for 20 h (LB) or 40 h (MM) at 37 °C. To calculate mutant frequencies, the number of mutants on each selection plate was divided by the total number of plated cells. Occasional jackpot cultures were removed from the analysis. Average frequencies with standard error (SE) were determined using the statistical software program Prism (GraphPad).

2.3. dNTP pool measurements

dNTP pool size experiments were performed on mismatch-repair-proficient derivatives of the wild-type (NR10835, NR10836), *dcd* (NR12578), and *ndk* (NR15669) strains. The dNTPs were determined in exponential-phase cells grown in LB medium. Cells were harvested at OD_{660} = 0.5, and dNTP pool data were recorded using the DNA polymerase-based assay, as described [32]. A possible source of error in this assay, when using Klenow fragment as DNA polymerase, is its possible usage of GTP and CTP – when present at high concentration – leading to an overestimation of the dGTP and dCTP concentrations [33]. However, we have found this to be a significant source of error only when the CTP/dCTP or GTP/dGTP ratios exceed about 100 [11]. In *E. coli* B, which is typical of wild-type *E. coli* strains, the CTP/dCTP and GTP/dGTP ratio do not exceed 10 [34]. Therefore, any errors resulting from the use of Klenow fragment to generate the current dNTP pool data are assumed to be insignificant.

3. Results

3.1. Mutability and mutational specificity

The purpose of our studies was to investigate the correlations of the cellular dNTP pools (and changes therein) with the cellular mutation rate or, more precisely, with the rate by which cells produce specific DNA replication errors. As replication errors are normally subject to correction by the postreplicative mismatch repair system (*mutHLS* system), which imposes its own specificity on the individual errors (different errors are corrected with different efficiency), we performed our mutagenesis experiments in strains defective in mismatch repair (*mutL*). In this manner, the observed mutations may be assumed to reflect directly the rate and specificity of the replication error production. In addition, we investigated the specificity of mutagenesis (types of errors) using the set of *lacZ* reversion alleles developed by Cupples and Miller [29]. This set of six *lacZ* missense alleles can each revert to *lac*$^+$ by one of the six possible base pair substitutions: A·T→C·G, G·C→A·T, G·C→C·G, G·C→T·A, A·T→T·A, and A·T→G·C [29]. In addition, we measured mutations resulting in resistance to the antibiotic rifampicin, for which mutations occur at a larger number of sites in the *rpoB* gene (forward system) [35].

3.2. dcd strains are mutators

The results presented in Table 1 show that a strain defective in dCTP deaminase (*dcd*) possesses elevated mutation rates, *i.e.*, *dcd* strains are mutators. This mutator effect has a clear specificity: when calculating the increase in mutant frequency for each of the six *lacZ* alleles, the increase is most pronounced for the *lac* G·C→T·A and A·T→T·A transversions, 27- and 42-fold, respectively, compared to the control *mutL* strain. In contrast, the increase using the Rif forward assay is only very modest, around 2-fold.

Table 1
Mutational effects and mutational specificity of *mutL* and *mutL dcd* strains (mutants per 10^8 cells).

Mutation	*mutL*	*mutL dcd*	*dcd* mutator effect
lac A·T→C·G	1.3 ± 0.3	4.0 ± 0.4	3.0
lac G·C→A·T	240 ± 85	1200 ± 280	5.0
lac G·C→C·G	≤0.2	≤0.8	–
lac G·C→T·A	8.0 ± 1.5	212 ± 94	27
lac A·T→T·A	3.5 ± 2.1	148 ± 54	42
lac A·T→G·C	21 ± 8	53 ± 10	2.5
Rif^r	126 ± 79	256 ± 192	2.1

The results are the average ± S.E. for three independent experiments, each using 12–15 cultures. The *dcd* mutator effect is calculated as the increase in frequency in *mutL dcd* strains compared to the corresponding *mutL* strain.

3.3. The mutator effect of ndk strain

The mutator effect of *ndk* strains has been reported before [22,23]. Nevertheless, the strain was included in the present study to permit analysis of the mutational specificity in our strains, to be used alongside the simultaneously determined dNTP pool changes (see below).

In Table 2, we list the results for the *ndk mutL* strain along with its *mutL* parent strain. The results show a strong mutator effect for the *lac* A·T→T·A transversion, but no or only small effects for the other *lac* alleles. In contrast, a clear 10-fold increase is observed for the rifampicin-resistant forward mutations. These results are similar to those observed by Miller et al. [23].

3.4. dNTP pool measurements

In order to best correlate the observed mutational effects with dNTP pool changes, the pools were measured in cells growing in LB medium (mutant frequencies were determined from cells growing in LB). The results for a large number of measurements in the wild-type strain show that, under these conditions, dCTP possesses the largest pool (75 pmoles/10^8 cells), whereas the smallest is observed for dGTP (8.0 pmoles/10^8 cells) (see Table 3). In the *dcd* strain, there is a strong further increase of the dCTP pool (near 4-fold) and a reduction in the dGTP concentration (2-fold). The elevation of the dCTP pool, also reported in early studies [18,19], is as expected in view of the lack of dCTP deaminase activity. No significant decrease of the dTTP pool [18,19] was found in our experiments. This is presumably due to the use of LB medium, which includes nucleotide precursors such as thymine and thymidine, which permit synthesis of dTTP *via* salvage pathways [36]. We did observe significant reductions in the dTTP pool when the *dcd* cells were grown in defined minimal media (not shown), consistent with the previous reports [18,19].

Table 2
Mutational effects and mutational specificity of *mutL* and *mutL ndk* strains (mutants per 10^8 cells).

Mutation	*mutL*	*mutL ndk*	*ndk* mutator effect
lac A·T→C·G	0.60 ± 3	0.68 ± 0.21	1.1
lac G·C→A·T	58 ± 6	100 ± 30	1.7
lac G·C→C·G	≤0.2	≤0.3	–
lac G·C→T·A	3.1 ± 0.7	5.8 ± 0.8	1.9
lac A·T→T·A	0.62 ± 0.12	15 ± 3	24
lac A·T→G·C	16 ± 3	40 ± 1	2.5
Rif^r	380 ± 55	3900 ± 1300	10

The results are the average ± S.E. for three independent experiments, each using 12–15 cultures. The *ndk* mutator effect is calculated as the increase in frequency in *mutL ndk* strains compared to the corresponding *mutL* strain.

Table 3
dNTP pools in wild-type, *ndk*, and *dcd* strains (pmoles/10^8 cells).

Strain	Genotype	Expts	dATP	dTTP	dCTP	dGTP
NR10835	wt	17	16 ± 7	15 ± 6	75 ± 19	8.0 ± 2.4
NR12578	*dcd*	6	11 ± 5	13 ± 7	**277 ± 39 (3.7)**	**3.8 ± 1.5 (2.0)**
NR15669	*ndk*	12	**4.0 ± 1.8 (0.25)**	23 ± 7	**150 ± 18 (2.0)**	7.5 ± 1.7
JC7623	wt	6	17 ± 9	51 ± 25	42 ± 22	10 ± 5
QL7623	*ndk*	6	11 ± 4	60 ± 26	**250 ± 80 (6.0)**	**25 ± 9 (2.5)**

The data are the mean (±SE) for the indicated number of independent measurements (experiments). In parentheses, statistically significant changes (fold) relative to the wild-type (also **bold** type). All strains were grown in LB medium. dNTP pools were measured as described in Section 2.

In the *ndk* strain, we observe two types of effect, a significant reduction in the dATP pool (4-fold) coupled with a doubling of the dCTP pool (Table 3). For comparison, we also determined again the pools for the previously described QL7623 (*ndk*) strain and corresponding wild type (JC7623) [22]. In this background, the *ndk* strain showed no significant decrease of the dATP pool; instead, a significant increase (6-fold) occurs in the dCTP level, as well as a modest increase (2.5-fold) for dGTP (Table 3). These results are more similar to the previously reported values for this strain background (a 20-fold increase in dCTP and a 7-fold increase in dGTP) [22]. It is to be noted that the JC and QL strains were *recB recC sbcB* strains used for the chromosomal deletion of the *ndk* gene and contain a further 21 known mutations (*E. coli* Genetic Stock Center). Thus, it may perhaps not be surprising that different dNTP pool effects are noted. The finding also highlights the fact that strain backgrounds, as well as growth media, need to be taken into account when investigating dNTP pools and associated effects.

4. Discussion

4.1. dNTP pools and polymerase error rate

From *in vitro* studies with DNA polymerases, it has become clear that DNA polymerase error rates depend on a number of variables [1,2]. At the nucleotide insertion step, each DNA polymerase has an intrinsic error rate, which represents the probability that it may insert a wrong nucleotide at a given template position. Insertion error rates generally vary with the type of error and also with the DNA sequence context. Broadly, transition errors (purine·pyrimidine mispairings, like T·G or A·C) are made more frequently than transversion errors (pyrimidine·pyrimidine or purine·purine mispairings). Importantly, since correct and incorrect dNTPs compete with each other at the nucleotide insertion step, a linear correlation is expected between the incorrect/correct dNTP ratio and the misinsertion rate. Thus, independent of the magnitude by which a DNA polymerase may discriminate against insertion of a particular incorrect nucleotide (intrinsic misinsertion fidelity), an *x*-fold change in the incorrect/correct ratio should lead to a corresponding *x*-fold change in the misinsertion rate.

A second critical aspect of dNTP pools regarding polymerase fidelity relates to the subsequent editing (proofreading) step, whereby the polymerase has an opportunity to excise the newly misinserted base. In *E. coli* this step is performed by the Pol III ε subunit (*dnaQ* gene product) [37,38]. The fidelity contribution of the proofreading step *in vivo* is substantial (up to two orders of magnitude), based on the strong mutator effects associated with defective *dnaQ* mutants [38]. As judged from polymerase studies *in vitro*, the efficiency of proofreading can be affected by the dNTPs, in particular those that are to be inserted directly following the mismatch: higher "next" dNTPs will act to promote extension of the mismatch at the expense of the reverse excision step [1,39]. Higher dNTPs can therefore be mutagenic, and this "next nucleotide" effect is likely to operate *in vivo* as well [12,13]. The combined effects of the currently observed dNTP changes on both polymerase misinsertion and proofreading are discussed below.

4.2. Mutation rates and dNTP pool changes in dcd mutants

Our results show that *dcd* mutants display a specific enhancement of the dCTP pool (Table 3). At the same time, the associated mutational data (Table 1) show a specific enhancement of the *lac* G·C→T·A and A·T→T·A transversions (27- to 42-fold). In the scheme of Fig. 2 we analyze the six *lacZ* base substitutions in terms of the two possible mispairing errors that can lead to each substitution, as well as the next nucleotide to be incorporated for each case. The format used to describe each mispairing event is: "template·(incorrect/correct dNTP)". For an optimal analysis one would like to know which of the two possible mispairs (1 or 2 in the scheme) is the more frequent one, because, obviously, an increase in error rate predicted for the predominant mispair will be readily observable as an increase in the mutation rate, whereas such an effect for the minor mispair could remain obscured. The predominant event for at least four of the *lac* base substitutions appears well established [40–42], and these are **bolded** and **boxed** in the diagram: G·T and T·G mispairs for the two transitions (G·C→A·T and A·T→G·C, respectively) and the T·T and C·T pyrimidine·pyrimidine mispairs (G·C→T·A and A·T→T·A transversions, respectively) [40–42]. As in the *dcd* strain dCTP is the nucleotide that is primarily affected, we have it presented in color: *red*, when its enhancement is predicted to mutagenic, and *blue* when expected to be antimutagenic (likewise for dGTP, which is 2-fold reduced). As an example (Fig. 2), the analysis shows that the *lac* G·C→A·T transition would be disfavored at the insertion step (because dCTP is the correct nucleotide at this site), but would be promoted at the extension step (dCTP is the next nucleotide). A modest 5-fold increase in mutant frequency is observed (Table 1),

lac allele	Substitution		Mispair 1	Next dNTP		Mispair 2	Next dNTP
CC101	A·T→C·G		A·(**G**/T)	dATP		T·(**C**/A)	dATP
CC102	G·C→A·T		G·(T/**C**)	**dCTP**		C·(A/**G**)	**dGTP**
CC103	G·C→C·G		G·(**G**/**C**)	dATP		C·(**C**/**G**)	dATP
CC104	G·C→T·A		G·(A/**C**)	**dGTP**		C·(T/**G**)	**dCTP**
CC105	A·T→T·A		A·(A/T)	**dGTP**		T·(T/A)	**dCTP**
CC106	A·T→G·C		A·(**C**/T)	dATP		T·(**G**/A)	dATP

Fig. 2. Predicted effects of measured dNTP changes in the *E. coli dcd* mutant on replication errors. Shown are the 12 possible mispairing errors for the six different base substitutions that can be scored in the *lacZ* reversion system [29] as well as the (next) dNTP to be inserted immediately following the mispair. The format for the mispairs is: template base (wrong base/correct base). In *red*, we denote dCTP or dGTP changes that are predicted to promote the indicated errors, either by favoring misinsertion or by promoting mispair extension; in *blue*, dCTP or dGTP changes that would disfavor the errors. The wild-type DNA sequence at the site of *lac* reversion is 5′-AAT GAG AGT-3′, while in strains CC101 through CC106 the GAG codon (encoding the essential glutamic acid residue) is replaced by TAG, GGG, CAG, GCG, GTG, or AAG, respectively [29]. Only the single-base change to GAG will restore the Lac^+ phenotype. The preferred mispairings for four of the six base-pair substitutions are bolded and boxed. See text for further details.

lac allele	Substitution		Mispair 1	Next dNTP		Mispair 2	Next dNTP
CC101	A·T→C·G		A·(G/T)	dATP		T·(C/A)	dATP
CC102	G·C→A·T		G·(T/C)	dCTP		C·(A/G)	dGTP
CC103	G·C→C·G		G·(G/C)	dATP		C·(C/G)	dATP
CC104	G·C→T·A		G·(A/C)	dGTP		C·(T/G)	dCTP
CC105	A·T→T·A		A·(A/T)	dGTP		T·(T/A)	dCTP
CC106	A·T→G·C		A·(C/T)	dATP		T·(G/A)	dATP

Fig. 3. Predicted effects of measured dNTP changes in the *E. coli ndk* mutant on replication errors. Shown are the twelve possible mispairing errors for the six different base substitutions that can be scored in the *lacZ* reversion system [29] as well as the (next) dNTP to be inserted immediately following the mispair. In *red*, we denote dATP or dCTP changes that are predicted to promote the indicated errors, either by favoring misinsertion or by promoting mispair extension; in *blue*, dATP or dCTP changes that would disfavor the errors. See legend to Fig. 2 and text for further details.

not inconsistent with the two opposing effects. Importantly, a straightforward mutator effect is predicted for the G·C→T·A and A·T→T·A transversions, as dCTP is the next nucleotide in both cases. No particular effect is predicted for the A·T→G·C transition, consistent with the observation. It is possible that the weak 2.5-fold effect for this transition results from an enhancement of the minor A·C mismatch. Finally, the 3-fold increase in the A·T→C·G transversions may be due to increased T·C mispairing, although this remains speculative. Overall, it can be concluded that the *lacZ* reversion system is an informative tool to investigate the correlations between dNTP pools and replication error rates. We also wish to highlight that a 4-fold increase in the dCTP concentration leads to a 30- to 40-fold in the observed error rate, signifying that *in vivo* exonucleolytic proofreading is a powerful fidelity instrument. The lack of a significant *dcd* effect on the rifampicin-resistant mutants is discussed in Section 4.4.

4.3. Mutation rates and dNTP pool changes in ndk mutants

The pool changes in *ndk* are distinct from that of *dcd* and, hence, mutational consequences are expected to be different, as is indeed observed (Tables 1 and 2). In the *ndk* mutant there is a 2-fold enhancement of the dCTP pool and a 4-fold decrease in the dATP pool (Table 3). Continuing the logic developed above, we use the scheme of Fig. 3 to make possible correlations between the mutational specificity and pool changes. It is clear that the strong *ndk* mutator effect for the A·T→T·A transversions can be rationalized by the decrease in dATP (correct nucleotide) and simultaneous increase in dCTP (next nucleotide). None of the other mispairings is subject to such a double promotion, and this is fully consistent with the unique enhancement of the A·T→T·A transversions. The remaining, modest frequency increases for the G·C→A·T, G·C→T·A and A·T→G·C substitutions are consistent with a single modest effector (2-fold enhancement of dCTP) or a combination of a positive and negative effects.

4.4. Mutational specificity in the rpoB target

The correlations between dNTP pool changes and mutational specificity can also be investigated using the rifampicin-resistance marker, which yields mutations in the *rpoB* gene rendering RNA polymerase resistant to rifampicin. Our data show an only very modest (2-fold) mutator effect for the *dcd* mutant, but a strong (at least 10-fold) for the *ndk* mutant. While we have not sequenced the rifampicin-resistant mutants in the present study, conclusions on this issue can be readily drawn based on the studies by Miller et al. [23] and Garibyan et al. [35], which provide detailed information on the types of mutations and their sites of occurrence in the *rpoB* gene in the MMR-defective background, including the events specifically enhanced by the *ndk* mutator [23].

The main hotspot for *rpoB* mutants in the MMR-defective *ndk* strain is an A·T→G·C transition at position 1547 (22 events out of 33 total) [23]. This is also the main hotspot in the single MMR-defective strain (29 out of 57) and therefore reflects the occurrence of the most frequent DNA replication error. The local sequence context for position 1547 is 5′-GTC-3′ (1547 underlined). Assuming that the A·T→G·C at this position reflects $T_{template}$·G mispairing, the incorrect dGTP competes at the misinsertion step with the correct dATP, while dCTP is the next nucleotide. It is clear from the earlier analysis that both *ndk*-mediated dNTP pool changes (lower dATP, higher dCTP) will cooperate to drive this event. The second *rpoB* hotspot (position 1534) induced by *ndk* is also a 5′-GTC-3′ sequence and its increase can be similarly accounted for. We note here that, as opposed to the two indicated hotspots in *rpoB*, the A·T→G·C transition in the *lac* system (Table 2), is not significantly elevated, as in this case dATP is the next nucleotide hence providing for an antimutator effect rather than mutator effect at the proofreading step (dATP is 4-fold down).

In contrast to the *ndk* strain, the *dcd* strain is not a strong mutator for rifampicin-resistant mutations. As a strong mutator effect can only be expected if there is an enhancement of the existing main events in the MMR-defective background, one would again be looking for enhancement of the A·T→G·C mutations at the 5′-GTC-3′ of positions 1547 and 1534 [23]. While the increase in dCTP would drive the extension reaction, the $T_{template}$·G mispairing would not be enhanced and, in fact, would be reduced by the 2-fold lowering of the dGTP concentration (Table 3) (dGTP is the misinserting nucleotide). Thus, in order to enhance rifampicin-resistant mutations higher dCTP and either elevated dGTP or lowered dATP is needed. This requirement is met by the *ndk* mutator but not the *dcd* mutator.

4.5. General comments on in vivo dNTP pool changes

Our analysis of dNTP pool changes and associated mutational effects indicates that the rules for the dNTP-dependence of replication fidelity, as uncovered based on studies with isolated DNA polymerases, can be applied to the behavior of *in vivo* replicases. Our case concerns the bacterium *E. coli*, but similar assumptions have been applied to the yeast *S. cerevisiae* [13]. A notable feature that has emerged from our study is the strong fidelity role played by the 'next-nucleotide' dNTP: strong mutagenic effects are observed due to increased levels of dCTP in both the *lac* and *rpoB* systems when it serves as a next nucleotide. This is not only consistent with the notion that proofreading is an important fidelity *in vivo* mechanism [38,43], but also suggests that a main reason for cells to keep the dNTP concentrations low (in the micromolar level, as opposed to the millimolar level for the NTPs) is to be able to control the error rate of the replication process.

Our data and analysis yield plausible correlations between dNTP changes and resulting mutational consequences. These correlations support the previously proposed model that, indeed, this particular mutator effect may result from the observed dNTP pool changes [22]. This is important because it has been argued elsewhere that simple dNTP changes are not sufficient to explain the *ndk* mutator effect [24]. The strongest argument in favor of this viewpoint is results in which a clone of the human NDK homolog *nm23*-H2 was able to reduce (to near wild-type level) the frequency of rif^R mutants of an *ndk* strain [24] (as also demonstrated in [23]); however, when the dNTP pools were determined in the complemented strain, distorted pools could still be observed, seemingly unlinking the relationship between mutator effect and dNTP changes. However, caution is required with conclusions of this type. Correlations between dNTPs and mutations are complicated, requiring

interpretation in terms of both correct and incorrect nucleotides as well as next nucleotides, and while these correlations can be drawn satisfactorily as shown here, the correlations are not necessarily quantitative, particularly when considering the next-nucleotide effect. For example, a 4-fold increase in the dCTP concentration, as a next nucleotide, can be responsible for an up to 42-fold increase in A·T→T·A transversions (*dcd*, see Table 1, Fig. 2). Thus, while dNTP changes in *nm23-H2*-complemented *ndk* strains were judged to be small and possibly not significant [24], it cannot be excluded that they are, in fact, consistent with the observed loss of mutability. The conclusions of Nordman and Wright [24] are based on measurement of rifampicin-resistant mutants. As noted, these events, in *nkd* strains are dominated by A·T→G·C transitions at a few hotspot sites, in particular the two 5′-GTC-3′ sequences at positions 1534 and 1547 [23]. For these events, in addition to the increased dCTP (the next nucleotide), the dGTP/dATP (incorrect/correct) ratio appears to be a critical factor (see Section 4.4). Importantly, as complementation of *ndk* with the human NDK homolog *nm23*-H2 appeared to substantially restore the dATP concentration (from 0.27 to 0.76 of wild-type level) [24], this restoration may provide a plausible explanation for the reported loss of mutator activity.

As second aspect that requires consideration is the role of DNA mismatch repair (MMR). Our present studies were conducted in the MMR-defective strain background to focus directly on replication errors. The complementation studies of *ndk* with *nm23-H2* were conducted in both MMR-proficient and MMR-defective backgrounds [24]. While in the MMR^+ background, complementation led to loss of mutability, this was not the case in the MMR^- background (Table S1 in [24]). The latter fact clearly indicates that in the *nm23*-H2 complemented *ndk* strain the enhanced replication errors are still occurring, thus preserving the correlation between dNTP pool changes and mutability. These observations suggest that the functioning of the DNA mismatch repair system may be a critical factor in shaping the *ndk* mutator effect. It is to be noted that the efficiency of mismatch repair can be compromised, wholly or in part, by the occurrence of frequent mispairing errors [44–46], and as a consequence modest changes in error rates can have disproportional effects (both positive and negative) on the observed mutation rates. Thus, mismatch-repair proficient strains are not an optimal vehicle for studying the effects of altered dNTPs on replication fidelity.

As an alternative to the dNTP hypothesis it was postulated that the *ndk* mutator effect is due to elevated dUTP levels [24]. This proposal was based on the strongly increased mutability observed for *ndk dut* strains, which are additionally defective in dUTPase activity (*dut* gene product). While this high mutability is intriguing, no direct evidence for this hypothesis is yet available. For example, no dUTP levels have been measured or reported for these strains. In addition, dUTP is not generally considered a mutagenic compound. While dUTP can go readily into DNA in place of dTTP and, once in the DNA, can be excised by uracil glycosylase generating repair intermediates such as abasic sites, neither uracils nor abasic sites are considered to be particularly mutagenic, as evidenced, for example, by the lack of mutability of *dut* strains [47]. While abasic sites can be mutagenic under conditions of SOS-induction [48], this type of damage-dependent mutagenesis is not generally consistent with the A·T→G·C specificity observed in the *rpoB* (and *lacI*) gene target [24]. In view of the above, we suggest that the direct mispairing model remain the simplest, minimal hypothesis to explain the *ndk* mutator effect. Obviously, additional experimentation is required to settle these issues.

Conflict of interest statement

The authors declare that there are no conflicts of interest

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References

[1] T.A. Kunkel, DNA replication fidelity, J. Biol. Chem. 279 (2004) 16895–16898.
[2] T.A. Kunkel, K. Bebenek, DNA replication fidelity, Annu. Rev. Biochem. 69 (2000) 497–529.
[3] P. Nordlund, P. Reichard, Ribonucleotide reductases, Annu. Rev. Biochem. 75 (2006) 681–706.
[4] E.C. Friedberg, DNA Repair and Mutagenesis, 2nd ed., ASM Press, Washington, DC, 2006.
[5] M.J. Bessman, D.N. Frick, S.F. O'Handley, The MutT proteins or Nudix hydrolases, a family of versatile, widely distributed, housecleaning enzymes, J. Biol. Chem. 271 (1996) 25059–25062.
[6] M.Y. Galperin, O.V. Moroz, K.S. Wilson, A.G. Murzin, House cleaning, a part of good housekeeping, Mol. Microbiol. 59 (2006) 5–19.
[7] H. Maki, M. Sekiguchi, MutT protein specifically hydrolyses a potent mutagenic substrate for DNA synthesis, Nature 355 (1992) 273–275.
[8] L.J. Wheeler, I. Rajagopal, C.K. Mathews, Stimulation of mutagenesis by proportional deoxyribonucleoside triphosphate accumulation in *Escherichia coli*, DNA Repair 4 (2005) 1450–1456.
[9] C.K. Mathews, J. Ji, DNA precursor asymmetries, replication fidelity, and variable genome evolution, BioEssays 14 (1992) 295–301.
[10] S. Song, Z.F. Pursell, W.C. Copeland, M.J. Longley, T.A. Kunkel, C.K. Mathews, DNA precursor asymmetries in mammalian tissue mitochondria and possible contribution to mutagenesis through reduced replication fidelity, Proc. Natl. Acad. Sci. U.S.A. 102 (2005) 4990–4995.
[11] L.J. Wheeler, C.K. Mathews, Nucleoside triphosphate pool asymmetry in mammalian mitochondria, J. Biol. Chem. 286 (2011) 16992–16996.
[12] D. Ahluwalia, R.J. Bienstock, R.M. Schaaper, Novel mutator mutants of *E. coli nrdAB* ribonucleotide reductase: insight into allosteric regulation and control of mutation rates, DNA Repair 11 (2012) 480–487.
[13] D. Kumar, A.L. Abdulovic, J. Viberg, A.K. Nilsson, T.A. Kunkel, A. Chabes, Mechanisms of mutagenesis *in vivo* due to imbalanced dNTP pools, Nucleic Acids Res. 39 (2011) 1360–1371.
[14] S. Gon, R. Napolitano, W. Rocha, S. Coulon, R.P. Fuchs, Increase in dNTP pool size during the DNA damage response plays a key role in spontaneous and induced-mutagenesis in *Escherichia coli*, Proc. Natl. Acad. Sci. U.S.A. 108 (2011) 19311–19316.
[15] L. Wang, B. Weiss, *dcd* (dCTP deaminase) gene of *Escherichia coli*: mapping, cloning, sequencing, and identification as a locus of suppressors of lethal *dut* (dUTPase) mutations, J. Bacteriol. 174 (1992) 5647–5653.
[16] H. Hama, N. Almaula, C.G. Lerner, S. Inouye, M. Inouye, Nucleoside diphosphate kinase from *Escherichia coli*; its overproduction and sequence comparison with eukaryotic enzymes, Gene 105 (1991) 31–36.
[17] H. Hama, C. Lerner, S. Inouye, M. Inouye, Location of the gene (*ndk*) for nucleoside diphosphate kinase on the physical map of the *Escherichia coli* chromosome, J. Bacteriol. 173 (1991) 3276.
[18] J. Neuhard, E. Thomassen, Altered deoxyribonucleotide pools in P2 eductants of *Escherichia coli* K-12 due to deletion of the *dcd* gene, J. Bacteriol. 126 (1976) 999–1001.
[19] G.A. O'Donovan, G. Edlin, J.A. Fuchs, J. Neuhard, E. Thomassen, Deoxycytidine triphosphate deaminase: characterization of an *Escherichia coli* mutant deficient in the enzyme, J. Bacteriol. 105 (1971) 666–672.
[20] M.L. Lacombe, L. Milon, A. Munier, J.G. Mehus, D.O. Lambeth, The human Nm23/nucleoside diphosphate kinases, J. Bioenerg. Biomembr. 32 (2000) 247–258.
[21] Q. Lu, M. Inouye, Adenylate kinase complements nucleoside diphosphate kinase deficiency in nucleotide metabolism, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 5720–5725.
[22] Q. Lu, X. Zhang, N. Almaula, C.K. Mathews, M. Inouye, The gene for nucleoside diphosphate kinase functions as a mutator gene in *Escherichia coli*, J. Mol. Biol. 254 (1995) 337–341.
[23] J.H. Miller, P. Funchain, W. Clendenin, T. Huang, A. Nguyen, E. Wolff, A. Yeung, J.H. Chiang, L. Garibyan, M.M. Slupska, H. Yang, Escherichia coli strains (*ndk*) lacking nucleoside diphosphate kinase are powerful mutators for base substitutions and frameshifts in mismatch-repair-deficient strains, Genetics 162 (2002) 5–13.
[24] J. Nordman, A. Wright, The relationship between dNTP pool levels and mutagenesis in an *Escherichia coli* NDP kinase mutant, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 10197–10202.

[25] J. Nordman, A. Wright, Escherichia coli nucleoside diphosphate kinase mutants depend on translesion DNA synthesis to prevent mutagenesis, J. Bacteriol. 193 (2011) 4531–4533.
[26] R.M. Schaaper, B.N. Danforth, B.W. Glickman, Rapid repeated cloning of mutant *lac* repressor genes, Gene 39 (1985) 181–189.
[27] I.J. Fijalkowska, R.M. Schaaper, Effects of *Escherichia coli dnaE* antimutator alleles in a proofreading-deficient *mutD5* strain, J. Bacteriol. 177 (1995) 5979–5986.
[28] E.C. Siegel, S.L. Wain, S.F. Meltzer, M.L. Binion, J.L. Steinberg, Mutator mutations in *Escherichia coli* induced by the insertion of phage mu and the transposable resistance elements Tn5 and Tn10, Mutat. Res. 93 (1982) 25–33.
[29] C.G. Cupples, J.H. Miller, A set of *lacZ* mutations in *Escherichia coli* that allow rapid detection of each of the six base substitutions, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 5345–5349.
[30] J.H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1972.
[31] H.J. Vogel, D.M. Bonner, Acetylornithinase of *Escherichia coli*: partial purification and some properties, J. Biol. Chem. 218 (1956) 97–106.
[32] C.K. Mathews, L.J. Wheeler, Measuring DNA precursor pools in mitochondria, Methods Mol. Biol. 554 (2009) 371–381.
[33] P. Ferraro, E. Franzolin, G. Pontarin, P. Reichard, V. Bianchi, Quantitation of cellular deoxynucleoside triphosphates, Nucleic Acids Res. 38 (2010) e85.
[34] C.K. Mathews, Biochemistry of deoxyribonucleic acid-defective amber mutants of bacteriophage T4. 3. Nucleotide pools, J. Biol. Chem. 247 (1972) 7430–7438.
[35] L. Garibyan, T. Huang, M. Kim, E. Wolff, A. Nguyen, T. Nguyen, A. Diep, K. Hu, A. Iverson, H. Yang, J.H. Miller, Use of the *rpoB* gene to determine the specificity of base substitution mutations on the *Escherichia coli* chromosome, DNA Repair 2 (2003) 593–608.
[36] F.C. Neidhardt, R. Curtiss, *Escherichia coli* and Salmonella: Cellular and Molecular Biology, 2nd ed., ASM Press, Washington, DC, 1996.
[37] R.H. Scheuermann, H. Echols, A separate editing exonuclease for DNA replication: the epsilon subunit of *Escherichia coli* DNA polymerase III holoenzyme, Proc. Natl. Acad. Sci. U.S.A. 81 (1984) 7747–7751.
[38] S.A. Taft-Benz, R.M. Schaaper, Mutational analysis of the 3′→5′ proofreading exonuclease of *Escherichia coli* DNA polymerase III, Nucleic Acids Res. 26 (1998) 4005–4011.
[39] T.A. Kunkel, R.M. Schaaper, R.A. Beckman, L.A. Loeb, On the fidelity of DNA replication. Effect of the next nucleotide on proofreading, J. Biol. Chem. 256 (1981) 9883–9889.
[40] I.J. Fijalkowska, P. Jonczyk, M.M. Tkaczyk, M. Bialoskorska, R.M. Schaaper, Unequal fidelity of leading strand and lagging strand DNA replication on the *Escherichia coli* chromosome, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 10020–10025.
[41] I.J. Fijalkowska, R.M. Schaaper, P. Jonczyk, DNA replication fidelity in *Escherichia coli*: a multi-DNA polymerase affair, FEMS Microbiol. Rev. (2012).
[42] M. Maliszewska-Tkaczyk, P. Jonczyk, M. Bialoskorska, R.M. Schaaper, I.J. Fijalkowska, SOS mutator activity: unequal mutagenesis on leading and lagging strands, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 12678–12683.
[43] R.M. Schaaper, Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*, J. Biol. Chem. 268 (1993) 23762–23765.
[44] I.J. Fijalkowska, R.M. Schaaper, Mutants in the Exo I motif of *Escherichia coli dnaQ*: defective proofreading and inviability due to error catastrophe, Proc. Natl. Acad. Sci. U.S.A. 93 (1996) 2856–2861.
[45] K. Negishi, D. Loakes, R.M. Schaaper, Saturation of DNA mismatch repair and error catastrophe by a base analogue in *Escherichia coli*, Genetics 161 (2002) 1363–1371.
[46] R.M. Schaaper, Mechanisms of mutagenesis in the *Escherichia coli* mutator *mutD5*: role of DNA mismatch repair, Proc. Natl. Acad. Sci. U.S.A. 85 (1988) 8126–8130.
[47] B.K. Duncan, B. Weiss, Specific mutator effects of *ung* (uracil-DNA glycosylase) mutations in *Escherichia coli*, J. Bacteriol. 151 (1982) 750–755.
[48] R.M. Schaaper, L.A. Loeb, Depurination causes mutations in SOS-induced cells, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 1773–1777.