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Fragmentation of Replicating Chromosomes Triggered by Uracil in DNA

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The *dut* mutants of *Escherichia coli* fail to hydrolyze dUTP and thus incorporate uracil into their DNA, suffering from chromosomal fragmentation. The postulated mechanism for the double-strand DNA breaks is clustered uracil excision, which requires high density of DNA-uracils. However, we did not find enough uracil residues or excision nicks in the DNA of *dut* mutants to account for clustered uracil excision. Using a *dut recBC*(Ts) mutant of *E. coli* to inquire into the mechanism of uracil-triggered chromosomal fragmentation, we show that this fragmentation requires DNA replication and, in turn, inhibits replication of the chromosomal terminus. As a result, origin-containing sub-chromosomal fragments accumulate in *dut recBC* conditions, indicating preferential demise of replication bubbles. We propose that the basic mechanism of the uracil-triggered chromosomal fragmentation is replication fork collapse at uracil-excision nicks. Possible explanations for the low level terminus fragmentation are also considered.

Keywords: *dut*; uracil; *recBC*; double-strand breaks; chromosomal fragmentation

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Introduction

Uracil incorporation into DNA in place of thymine with its subsequent excision is a potent agent of genetic instability.[1,2] Uracil incorporation increases in conditions of insufficient dTTP biosynthesis (caused indirectly by folate deficiency or directly by thymidylate synthetase inactivation) and leads to death in cultured prokaryotic and eukaryotic cells[3] and to chromosomal instability in humans.[2,4] On the other hand, the vulnerability of the dTTP biosynthesis step in DNA replication has long been successfully exploited in anti-cancer and anti-microbial therapies that target tetrahydrofolate recycling.[5,6] Surprisingly, the mechanisms of uracil-induced genetic instability and cell death are yet to be studied at both the molecular and chromosomal levels.

One of the underlying reasons of the cell poisoning by DNA-uracil was proposed to be the futile cycle of uracil-DNA incorporation and removal, leading to long-lived single-strand gaps and, eventually, to double-strand breaks of unspecified nature.[4,7–9] In support of this idea, uracil residues[7,8] and abasic sites/single-strand breaks due to uracil excision[10] become detectable in DNA in conditions when the dTTP biosynthesis is inhibited. Moreover, Blount and colleagues[1] correlated high uracil content in human DNA with elevated levels of double-strand breaks, which they attributed to simultaneous excision of clustered uracil bases (Figure 1). Indeed, double-strand breaks are detectable *in vivo* when plasmids carrying closely spaced uracil bases in opposite strands are introduced into *Escherichia coli* cells.[11,12] Excision of clustered UV or oxidative lesions is similarly proposed to result in "enzymatic" double-strand breaks after DNA damaging treatments.[13–15] Moreover, the uracil-triggered chromosomal fragmentation was recently confirmed in the *dut* mutants of *E. coli*[16,17] that fail to hydrolyze dUTP[18] and, therefore, incorporate uracil into their DNA.[19] Thus, there is good evidence that insufficient dTTP production results in uracil incorporation into the chromosomal DNA with subsequent uracil excision that causes genetic instability due to formation and repair of double-strand DNA breaks.

The biosynthesis of the DNA precursor dTTP in *E. coli* (Figure 1) starts with deamination of dCTP by deoxycytosine deaminase (the Dcd protein) to generate dUTP, which is then promptly hydrolyzed

Abbreviations used: ExoIII, exonuclease III; UDG, uracil-DNA-glycosylase; Ts, temperature-sensitive.

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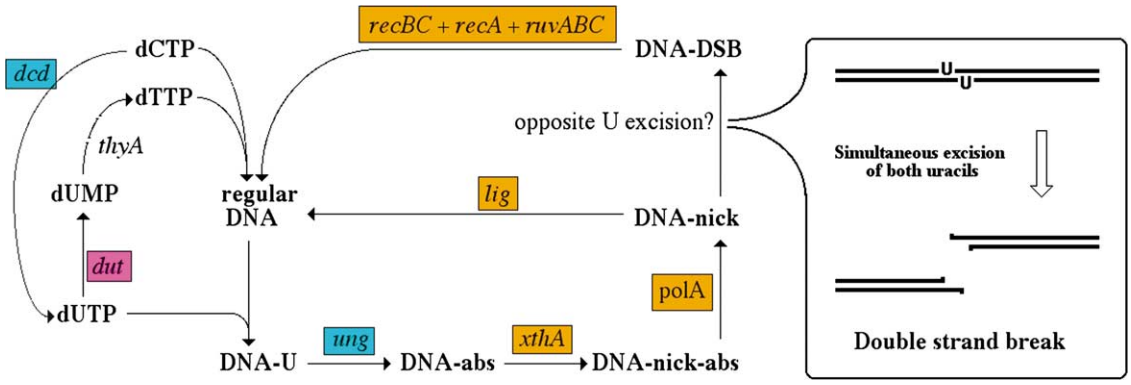

Figure 1. Metabolism of uracil in DNA: incorporation, excision and double-strand breaks. Left, the major pathways of dTTP production for the regular DNA synthesis and the consequences of uracil incorporation into DNA. The stage catalyzed by Dut is shown in magenta. Mutants inviable in *dut*-deficient background are shown in orange; mutants suppressing this synthetic lethality are shown in blue. Other enzymes, identified by their genes, are as follows: *dcd*, dCTP deaminase; *thyA*, thymidylate synthase; *ung*, uracil-DNA-glycosylase; *xthA*, exonuclease III; *polA*, DNA polymerase I; *lig*, DNA ligase. The substances are: dCTP, deoxycytidine triphosphate; dTTP, deoxythymidine triphosphate; dUMP, deoxyuridine monophosphate; dUTP, deoxyuridine triphosphate; DNA-U, DNA with uracil incorporated; DNA-abs, DNA with abasic sites; DNA-nick-abs, DNA with nicks at abasic sites; DNA-nick, DNA with nicks (the product of nick-translation by DNA pol I); DNA-DSB, DNA with double-strand breaks. Right, a scheme of formation of a double-strand DNA break due to simultaneous excision of clustered uracil bases.

to dUMP by dUTPase (the Dut protein).[20] In contrast, mammals derive their dUMP chiefly from dCMP.[21] From dUMP, the bacterial and the mammalian pathways coincide: using tetrahydrofolate cofactor, thymidylate synthetase (*thyA*) methylates dUMP to dTMP, which is then phosphorylated to dTTP.[20] It is important for the cell to keep dUTP concentration low, because dUTP is readily used by prokaryotic and eukaryotic DNA polymerases,[22–24] resulting in replacement of DNA-thymine with uracil. The major pathway to remove uracil from DNA (Figure 1) is initiated by uracil-DNA-glycosylase (the Ung protein in *E. coli*), while the resulting abasic sites are nicked by abasic site endonucleases (ExoIII and Endo IV in *E. coli*).[25] Other enzymes, such as EndoV of *E. coli*,[26] were also reported to attack uracil-containing DNA. Similar enzyme activities operate in eukaryotes to keep DNA free of uracil.[27]

Previously we observed chromosomal fragmentation in *E. coli dut recBC* mutants.[16,17] To detect double-strand breaks in *dut* mutants, inactivation of the RecBC enzyme is required, which blocks both the recombinational repair of the breaks and the degradation of the linear DNA.[28,29] The *dut recBC* double mutants cannot control chromosomal fragmentation and are synthetically lethal[16] (Figure 1). The primary cause of the fragmentation, which reaches 22% in four hours of growth in the double-mutant conditions (Figure 2),[17] is incorporation of uracil bases into DNA, because both the fragmentation and synthetic lethality in *dut recBC* mutants are suppressed by inactivation of *dcd* or *ung* genes[16] (Figure 1). Here we used the chromosomal fragmentation in *dut recBC* mutants to inquire into the mechanisms and consequences of uracil incorporation-triggered double-strand DNA breaks at the chromosomal level. Surprisingly, we found no evidence for clustered uracil excision. Instead, double-strand breaks arise in a replication-dependent manner, indicating that uracil excision causes replication fork collapse.

Results

Density of uracils in the DNA of *dut* mutants

The postulated mechanism for the double-strand breaks *via* simultaneous excision of opposite uracil bases (Figure 1)[1,11,12] requires high density of DNA uracil bases, which have to be present not only in the newly synthesized DNA strands, but also in the complementary "old strands", synthesized during preceding replication rounds. The previously reported amount of uracil in the chromosomal DNA of both the *dut* and *ung* mutants was below the level of detection of the radioisotopic analysis (<11,600 uracil bases per genome),[30] so we decided to use a more sensitive enzymatic assay. Plasmids are frequently used for DNA damage and repair studies *in vivo*, since it is generally assumed that plasmid DNA is subject to the same replication and repair dynamics as chromosomal DNA.[11,12,31–34] To estimate a steady-state level of uracil in the chromosomal DNA of *dut* and *ung* mutant cells relative to the wild-type cells we used a 22.5–kbp long supercoiled plasmid DNA isolated from the growing cultures of the corresponding strains and treated *in vitro* with UDG and ExoIII enzymes. A single uracil excision event converts a supercoiled molecule into the relaxed open circular one (Figure 3(a)). We take the amount of supercoiled plasmid DNA left after the enzymatic treatment for the zero class of the Poisson distribution to calculate