Uracil incorporation: A source of pulse-labeled DNA fragments in the replication of the Escherichia coli chromosome

(Okazaki fragments/dUTPase/uracil N-glycosidase/discontinuous DNA replication)

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ABSTRACT Uracil is incorporated into newly synthesized DNA by mutants of Escherichia coli with reduced levels of dUTPase (dUTP nucleotidohydrolase; EC 3.6.1.23). Excision-repair of the incorporated uracil results in the generation of labeled DNA fragments that appear after brief pulses with [3H]thymidine [Tye, B-K., Nyman, F.-D., Lehman, I. R., Hochhauer, S. & Weiss, B. (1977) Proc. Natl. Acad. Sci. USA 74, 154-157]. Uracil is also incorporated into the newly synthesized DNA of strains of E. coli that contain normal levels of dUTPase. DNA fragments generated by the postreplication excision-repair of uracil may therefore contribute to the pool of nascent DNA (Okazaki) fragments that normally appear in wild-type strains. Discontinuous DNA replication has been examined in the absence of uracil excision by comparing Okazaki fragments in strains that are defective in DNA polymerase I (polA−) and polA− strains that are also defective in uracil N-glycosidase, an enzyme required for the excision-repair of uracil in DNA (polA− ung−). Little or no difference was detected in the level of Okazaki fragments in the polA− strain as compared with the polA− ung− strain. Thus, the uracil-induced cleavage of DNA cannot be the sole mechanism for the generation of Okazaki fragments. Mutants that are defective both in dUTPase and in uracil N-glycosidase incorporate uracil into their DNA with a high frequency (up to 1 per 100 nucleotides). These uracil residues, once incorporated, persist in the DNA without an adverse effect on the growth of the cells.

Mutants of Escherichia coli defective in dUTPase (dUTP nucleotidohydrolase; EC 3.6.1.23) (dut− or sof−) accumulate labeled DNA fragments transiently after short pulses with [3H]thyminidine (1). These fragments result from the increased incorporation of uracil into nascent DNA as a consequence of the dUTPase defect, followed by excision-repair of the incorporated uracil. The repair-induced DNA fragments (Sof fragments) closely resemble nascent DNA (Okazaki) fragments (2). Both Okazaki fragments and Sof fragments appear transiently and are then rapidly chased into high molecular weight DNA. Both also rely on DNA polymerase I and DNA ligase for their subsequent joining into high molecular weight DNA (3-7). In fact, Sof fragments appear to act as precursors to Okazaki fragments in the appropriate pulse-chase experiments (7).

In the experiments to be reported here, we have sought to determine to what extent postreplication excision-repair of uracil in DNA contributes to the pool of Okazaki fragments observed in typical [3H]thyminidine pulse experiments. We have found that, as judged by the susceptibility in vitro to uracil N-glycosidase, an enzyme that excises uracil from DNA (8), uracil is indeed incorporated into pulse-labeled DNA even in the presence of normal levels of dUTPase. In view of the high efficiency with which uracil is removed from DNA in vivo (7), the uracil-induced cleavage of DNA should account for at least a part of the pool of Okazaki fragments.

MATERIALS AND METHODS

Bacterial Strains. The E. coli strains used were all derived from strain BD1154 (F− thi1, argH1, lacY1, galK6, malA1, xyl7, rha6, ara13, strA9, supE44, χ8) and contained additional mutations as follows: BD1157, ung1; BD1157, dUTPase (dUTP nucleotidohydrolase; EC 3.6.1.23); polA4113; polA4113 ung1; BKT406; polA4113; BKT411; polA4113 ung1; BKT404, polAex2; and BKT401, polAex2 ung1.

Preparation of Pulse-Labeled DNA. Cells were grown at 37 °C to an A950 of about 0.5 in minimal medium supplemented with 0.2% glucose, 0.5% casamino acids, 1.0 μg of thiamin per ml, and 2 μg of thymine per ml. Eighteen milliliters of culture were removed and pulsed at 37 °C for 10 sec with 400 μl of [3H]thymidine (20 Ci/mmol, 1 mM in saline, New England Nuclear). In experiments involving long-term labeling of cells (4 hr), the growth medium was further supplemented with 250 μg of deoxyadenosine per ml and [3H]thymidine was added in two portions, initially and at 2 hr. The pulses were terminated by addition of an equal volume of a solution composed of 75% ethanol/2% phenol/20 mM sodium acetate (pH 5.2)/2 mM EDTA. The resulting suspension was centrifuged and the pellet resuspended in 1.5 ml of 0.2 M NaOH/10 mM EDTA. Where indicated, 32P-labeled φX174 (10 μg of DNA (9)) was added as an internal control to monitor nonspecific hydrolysis. The mixture was incubated at 37 °C for 1 hr, then centrifuged to remove cellular debris. The supernatant fluid was chilled to 4 °C, brought to pH 8 with 1 M HCl, and then dialyzed against 10 mM Tris (pH 8.0)/5 mM EDTA for 1 hr at 4 °C.

Treatment of DNA with Uracil N-Glycosidase. The reaction mixture (200 μl) contained 50 mM N-2-hydroxyethylpipеразин-N'-2-ethanesulfonic acid (Hepes) buffer (pH 7.8), 1.2 mM dithiothreitol, 0.038 mg of bovine serum albumin per ml, and 2 mM EDTA. [3H]Thymidine-labeled DNA was present at a final concentration of 1 mM (in nucleotides), and 5 units of purified uracil N-glycosidase (9) were added per μmol of DNA. Incubation was at 4 °C for 2 hr. (Under these conditions, 1 unit of uracil N-glycosidase releases 0.2 μmol of uracil from PBSi DNA in 2 hr.) The reaction was terminated by the addition of 0.1 ml of a solution composed of 0.2 M NaOH/0.8 M NaCl/4 mM EDTA. The mixture was then layered onto a 5-20% alkaline sucrose gradient and centrifuged at 5 °C for 15 hr at 32,000 rpm in a Beckman L265B ultracentrifuge in an SW41 rotor.

RESULTS

Uracil Incorporation into DNA of a dut− ung− Double Mutant. Introduction of a mutation in uracil N-glycosidase
FIG. 1. Persistence of uracil in pulse-labeled DNA from the dut ung 1 double mutant. The dut ung 1 double mutant (BD1157) was pulse-labeled with [3H]thymidine at 37°C for 10 sec. The isolated DNA was treated with uracil N-glycosidase and sedimented in a 5-20% alkaline sucrose density gradient. The reaction mixture without uracil N-glycosidase was incubated and centrifuged in the same way.

(ung-') suppresses the Sof phenotype of dUTPase-defective (dut-) strains of E. coli, e.g., the transient accumulation of labeled fragments after brief [3H]thymidine pulses. Thus, the labeling pattern observed in the dut-ung- double mutant is indistinguishable from that seen in a wild-type strain pulsed under the same conditions (7). Similar suppression of the Sof phenotype can be demonstrated in vitro with the use of the purified uracil N-glycosidase. Pulse-labeled DNA was isolated from the dut-ung- mutant, denatured, and treated with uracil N-glycosidase. Excision of uracil results in the generation in the DNA of an apyrimidinic site sensitive to alkaline cleavage (10). Consequently, alkaline sucrose density gradient sedimentation of the N-glycosidase-treated DNA should provide an estimate of the amount of uracil present in the pulse-labeled DNA. Treatment of pulse-labeled DNA from the dut-ung- double mutant with uracil N-glycosidase followed by alkaline sucrose gradient sedimentation of the product resulted in the generation of the 4S fragments typical of those observed in vitro upon pulse labeling of the dut- mutant (Fig. 1). Thus, uracil has been incorporated into the DNA of the dUTPase-defective strain and persists as a result of the ung- mutation. The 4S fragments that appear after [3H]thymidine pulses of dut- strains in vivo must therefore be a consequence of the action of uracil N-glycosidase on the uracil-containing DNA generated by the dut- mutation. Direct demonstration of [3H]uracil incorporation in the DNA of a dut-ung- double mutant has been reported by Warner and Duncan (11).

Persistence of Uracil in DNA of the dut-ung- Double Mutant. Uracil incorporated into the DNA of the dut-ung- double mutant in [3H]thymidine pulse experiments persists for
at least the length of the pulse period (10 sec) (Fig. 1). To determine whether uracil is retained for longer periods, we labeled the dut-ung- mutant with [3H]thymidine during 4 hr of growth. The labeled DNA was isolated, treated with uracil N-glycosidase, and sedimented in an alkaline sucrose density gradient. The labeled DNA, most of which was initially of high molecular weight (>30 S), was degraded to 4S fragments upon treatment with uracil N-glycosidase (Fig. 2). Thus, uracil persists in DNA through several generations in the dut-ung- double mutant.

Uracil Incorporation into DNA of ung- and ung+ Strains. DNA isolated from an ung- mutant after a 10-sec pulse with [3H]thymidine shows an alkaline sucrose density gradient profile very similar to that observed for ung+ (wild type) cells pulsed under the same conditions. Approximately 30% of the label sedimented very rapidly (>30 S) and the remainder had an average sedimentation coefficient of 8-14 S. Upon treatment of the DNA with uracil N-glycosidase (followed by alkaline sucrose density gradient sedimentation), essentially all the rapidly sedimenting DNA was degraded to fragments with sedimentation coefficients in the range of 8-16 S (Fig. 3). A much less marked effect was observed with DNA from the wild-type (ung+) strain (Fig. 4). (The sedimentation properties of 32P-labeled φX174 DNA, which lacks uracil and which served as a control in these experiments, remained unchanged after treatment with uracil N-glycosidase.) Thus, uracil is incorporated into DNA even in the presence of normal levels of dUTPase and then is rapidly excised by the action of uracil N-glycosidase.

Okazaki Fragments in polA- and polA-ung- Mutants. Essentially all newly synthesized DNA in polA mutants appears in the form of 8-10S Okazaki fragments (5, 6). Since the Sf phenotype of dut- mutants can be suppressed by an ung- mutation (7), we wished to determine to what extent the Okazaki fragments that accumulate after comparable pulses of polA mutants can be similarly suppressed by the ung- mutation. Two different polA- ung- double mutants were constructed from E. coli polA2 (12) and E. coli polA4113 (13), both of which are temperature-sensitive, conditionally lethal mutants. The polA- strains were pulse-labeled at 44° with [3H]thymidine and the corresponding polA- ung- strains were pulse-labeled with [14C]thymidine. DNA from two strains was mixed and sedimented in an alkaline sucrose gradient. The sedimentation profiles of the polA- or polA- ung- pulse-labeled DNAs were similar, with the polA- ung- DNA sedimenting somewhat more rapidly than that from the polA- strains (Fig. 5). Because of the relatively weak dependence of sedimentation coefficient on molecular weight (14), a more sensitive method is required to determine the actual difference in size between the two populations of DNA fragments. Thus, in contrast to Sf fragments, the level of Okazaki fragments that accumulate in polA- mutants is not diminished by a mutation in uracil N-glycosidase (7).

DISCUSSION

Uracil is present in the newly synthesized DNA of strains of E. coli that contain normal levels of dUTPase but are defective in uracil N-glycosidase (ung-). In contrast, there is little if any uracil in the DNA of wild-type (ung+) strains. Thus, incor-
FIG. 5. Comparison of sedimentation profiles of pulse-labeled DNA from polA" and polA"ung" mutants. Mutant cultures were grown at 30° to an A596 of approximately 0.5. Six milliliters of culture was removed and equilibrated at 44° for 5 min before being pulsed with thymidine. The polA"ung" cultures, i.e., BKT401 (polAex2, ung 1) and BKT411 (polA113, ung 1), were pulsed at 44° for 10 sec with 150 μl of [14C]thymidine (58 mCi/mmol, 0.1 mCi/ml). The polA" cultures, i.e., BKT404 (polAex2) and BKT406 (polA113), were pulsed at 44° for 10 sec with 100 μl of [3H]thymidine (30 Ci/mmol, mCi/ml). After the pulses, the cells were centrifuged and lysed by suspending the pellets in 0.6 ml of 0.2 M NaOH/10 mM EDTA. Twenty microliters of each of the polA" lysates and 250 μl of the polA"ung" lysates were mixed and sedimented in a 5–20% alkaline sucrose density gradient. 32P-Labeled φX174 DNA served as a 16S sedimentation marker. Sedimentation profiles are plotted as percent of total counts in each fraction.

Poration of uracil into DNA and its subsequent excision are processes that occur normally in E. coli. Since treatment of the DNA with uracil N-glycosidase in vitro results in a decrease in sedimentation coefficient from approximately 30 S to 8–16 S, uracil incorporation probably occurs on an average of one per 2000–5000 nucleotides. A recent analysis of several of the kinetic parameters of a nearly homogeneous dUTPase from E. coli by Shlomai and Kornberg indicates that an intracellular dUTP concentration of 0.5 μM is required to generate the dUMP required for normal rates of dTTP and DNA synthesis. Inasmuch as DNA polymerase III holoenzyme (15) has the same Km for dUTP and dTTP, this level of dUTP in the presence of an intracellular dTTP concentration of 150 μM would lead to uracil incorporation into DNA at a frequency of 1 per 300 thymines or 1 per 1200 nucleotides. This analysis, taken together with the findings reported here, strongly indicate that a portion of the Okazaki fragments normally seen in [3H]thymidine pulse labeling experiments originates from postreplication repair of uracil-induced damage to DNA. It is, however, also clear that this process cannot be the sole mechanism for the generation of Okazaki fragments.

It is not known whether replication of the E. coli chromosome proceeds discontinuously on only one of the two strands at the replication fork or whether replication is discontinuous on both strands (see ref. 16 for a discussion of this point). The finding that essentially all of the pulse-labeled DNA that appears in polA"ung" as well as in polA" mutants is in the form of 8–10S fragments would suggest that if DNA replication is discontinuous on only one strand, then repair mechanisms other than that after uracil incorporation result in fragmentation of the strand that is synthesized continuously. Alternatively, DNA replication may occur discontinuously on both strands.

Very recently, Olivera (17) has found that replication of the E. coli chromosome in an in vitro system, i.e., concentrated cell lysates deposited on cellophane discs (18), proceeds discontinuously on only one of the two strands at the replication fork.

The finding that a mutant defective in both dUTPase and uracil N-glycosidase retains uracil in its DNA through several generations suggests that uracil N-glycosidase is the enzyme most responsible for the removal of uracil residues incorporated into DNA. Endonuclease V, also known to recognize uracil in DNA and to cleave phosphodiester bonds at or near these residues (19), is probably of lesser significance in the removal of uracil paired with adenine; it may participate in the excision of uracil produced by the deamination of cytosine in DNA and therefore mismatched with guanine (8).

The dut"ung" double mutant appears to be largely unaffected by levels of uracil in its DNA up to 1 per 100 nucleotides. A similar result has been reported by Warner and Duncan (11). We have not yet determined the effects of more extensive substitution of uracil for thymine in E. coli.

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