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EXCISION REPAIR OF URACIL DURING REPLICATION OF ØX174 DNA IN VITRO

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SUMMARY: Each of the stages in the replication of ØX174 DNA in vitro, e. g., conversion of circular single stranded parental DNA to the duplex replicative form (SS → RF), replication of the closed circular duplex form (RF → RF), and synthesis of circular single stranded progeny DNA (RF → SS), may be affected by a reduced level of dUTPase. Thus, in enzyme preparations from mutant strains defective in dUTPase (dut⁻), the complementary strand synthesized in the SS → RF reaction is abnormally short (7-8S vs. 14S), and the extent of RF replication is decreased 10-fold. Preferential removal of dUTPase during fractionation of enzyme preparations from wild type (dut⁺) cells may produce comparable effects. In particular, the single stranded circular DNA synthesized in the RF → SS reaction by a set of highly purified enzymes is rapidly degraded upon incubation with the less pure enzymes required for its conversion to RF. All of these effects are plausibly accounted for by the incorporation into DNA of uracil from dUTP, possibly present as a contaminant in one or more components of the reaction, followed by excision of the uracil and phosphodiester bond cleavage at the resulting apyrimidinic site.

INTRODUCTION

Mutants of Escherichia coli with a defect in dUTPase (dut⁻ or sof⁻) transiently accumulate labeled DNA fragments after brief pulses with [^{3}H]thymidine (1). These fragments are kinetically indistinguishable from the Okazaki fragments that appear following comparable pulses of cells defective in DNA ligase or DNA polymerase I, and 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 (2).

Because of the striking effect of uracil excision-repair on DNA replication in vivo, we wished to determine whether there were comparable effects on DNA replication in vitro. Since all three stages in the replication of ØX174 DNA can be reproduced in vitro, e. g., conversion of single stranded parental

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DNA to the duplex replicative form (SS → RF) (3, 4), replication of the closed circular duplex form (RF → RF) (5, 6), and synthesis of single stranded progeny DNA (RF → SS) (7), we examined each of these stages using enzyme preparations from wild type (dut$^+$) and dut$^-$ cells. All of them were strongly influenced by a reduced level of dUTPase.

MATERIALS AND METHODS

Bacterial Strains. Strains KS474 (dut$^+$) and RS5087 (dut 11) have been described (2)

Nucleic Acids. ØX174 SS and RFI DNAs were isolated according to Eisenberg et al. (5). ^{3}H-labeled ØX174 SS DNA synthesized in vitro (7) was kindly given to us by Dr. Shlomo Eisenberg.

ØX174 SS → RF and RF → RF syntheses were performed as described by Wickner et al. (3) and by Eisenberg et al. (5), respectively. Alkaline sucrose density gradient sedimentation was performed according to Tye et al. (1).

Nucleotides. Unlabeled dNTPs were obtained from PL Chemicals, [^{32}P]dCTP from ICN, and [5-^{3}H] dUTP from Amersham-Searle.

Enzymes. Uracil N-glycosidase (8) was kindly provided by Drs. Bruce Duncan and Huber Warner. The ØX174 cisA protein was a gift from Dr. Shlomo Eisenberg. Preparation of an E. coli enzyme fraction (Fraction II) capable of promoting the conversion of ØX SS → RF and ØX RF → RF was according to the method of Eisenberg et al. (5).

RESULTS

Conversion of ØX174 SS to RF. Incubation of ØX174 SS DNA with a partially purified E. coli enzyme preparation (Fraction II, see Materials and Methods) resulted in its conversion to the duplex RFII (3, 4). The product was the full length linear complementary strand with a sedimentation coefficient of 14S. When this reaction was performed using an enzyme preparation from cells defective in dUTPase (dut$^-$), the rate and extent of synthesis were the same as in the wild type, however, the sedimentation coefficient of the product was only 8S (Fig. 1). The approximately five-fold decrease in chain length was not the result of non-specific nuclease action. Thus, when ^{3}H-labeled ØX174 SS DNA was used as template in the presence of [^{32}P] dCTP, the sedimentation coefficient of the ^{32}P-labeled product was 7S, whereas the ^{3}H-labeled ØX174 SS DNA which served as template remained unchanged at 16S (Fig. 2).

The 7-8S product synthesized by the dut$^-$ preparation had a density characteristic of RFII as judged by CsCl density gradient centrifugation (Fig. 3). Thus, the RFII contained multiple nicks or gaps in the product strand rather than partially duplex DNA with extended single stranded regions. Inasmuch as both DNA polymerase I (or the T4-induced DNA polymerase) and DNA ligase were required to generate a 14S full length linear molecule (data not shown), it is likely that short gaps rather than simple nicks separate the 7-8S fragments annealed to the single stranded ØX174 template.

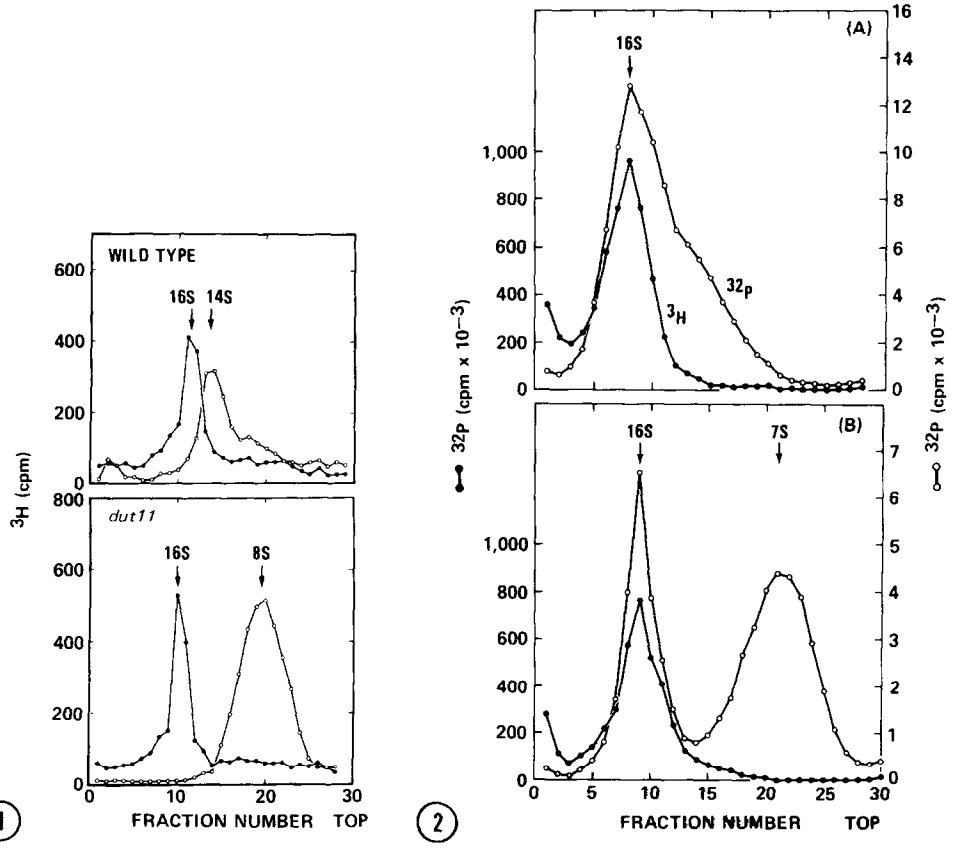

Figure 1: Synthesis of abnormally short product strands during ØX174 SS → RF conversion by an enzyme preparation from dut⁻ cells. ØX174 SS → RF synthesis using Fraction II and alkaline sucrose density gradient sedimentation of the product were performed as described by Wickner et al. (3) and by Tye et al. (1) respectively.

Figure 2: Preferential cleavage of product strand during ØX174 SS → RF conversion by enzyme preparations from dut⁻ cells. ØX SS → RF synthesis using Fraction II preparations from dut⁺ (A) and dut⁻ (B) cells was performed as described by Wickner et al. (3) with the exception that 0.58 μg ^{3}H-labeled ØX174 SS DNA (8.6 x 10^3 cpm) was added as template. [^{32}P]dCTP was the labeled deoxynucleoside triphosphate, and ^{32}P-labeled ØX174 phage (1.5 x 10^4 cpm) was added as a marker.

Since the only difference between the extracts derived from the wild type and dut 11 cells is in their content of dUTPase (0.36 μmol as compared with 0.018 μmol of dUMP formed per min/mg protein (1)), the decrease in sedimentation coefficient of complementary strands synthesized by the dut⁻ enzyme preparation is very likely the result of incorporation of uracil from dUTP,

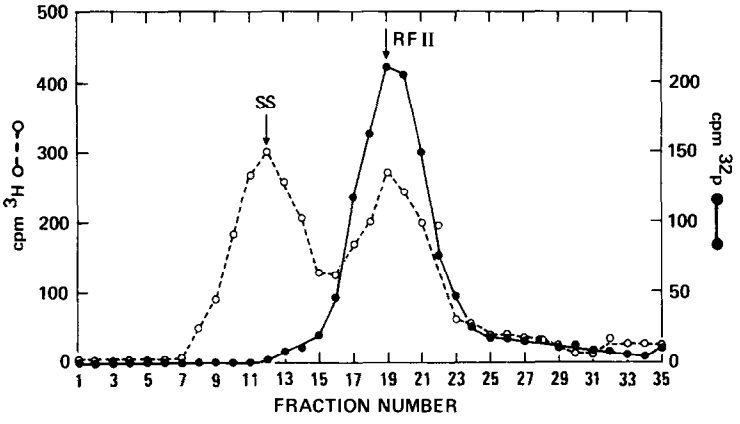

Figure 3: CsCl density gradient sedimentation of ØX174 RFII synthesized by an enzyme preparation from dut⁻ cells. ^{32}P-labeled ØX RFII was synthesized using Fraction II as described by Wickner et al. (3) with the exception that 10 µl of antibody to DNA polymerase I was added (1 µl gave > 95% inhibition of the activity of 0.15 pmol of homogeneous enzyme.). The product was chilled at 0° and a ^{3}H-labeled ØX RFII marker added. The mixture was made 25 mM in sodium pyrophosphate, 25 mM in EDTA and 0.52% in sodium dodecyl sulfate, and sedimented in a 5-20% neutral sucrose density gradient containing 1 M NaCl, 0.05 M Tris-HCl (pH 8.0), and 2 mM EDTA. Centrifugation was in a Beckman L265B centrifuge, with an SW56 rotor at 15° at 54,000 rpm for 2.6 hr. The fractions in which the ^{3}H-labeled ØX RFII marker and ^{32}P-labeled ØX RFII products were coincident and were pooled and sedimented in a CsCl density gradient containing 1.25 g CsCl per gram of solution. ^{3}H-labeled ØX SS DNA served as a marker. Sedimentation was in a Beckman L265B centrifuge using a fixed angle 65 rotor at 18° at 40,000 rpm for 46 hr. Sedimentation of the ^{32}P-labeled ØX RFII in an alkaline sucrose density gradient as in Figure 1 verified that the product consisted of 8S segments.

possibly present as a contaminant in one or more components of the reaction, followed by excision of the uracil by uracil N-glycosidase and subsequent nuclease action at the apyrimidinic site created. This conclusion is further supported by the finding that addition of dUTP to the reaction resulted in a decrease in sedimentation coefficient of the product strand. Thus, addition of dUTP at a concentration of 4 µM in the presence of 40 µM of each of the other deoxynucleoside triphosphates resulted in a change in sedimentation coefficient from 7S to 5.5S; at 40 µM dUTP, the sedimentation coefficient of the product was 3S. Similar findings have been reported by Olivera (9) and by Reichard (10) with other in vitro DNA replication systems.

In an experiment in which [^{3}H] dUTP was used in addition to the four deoxynucleoside triphosphates, the level of [^{3}H] uracil in the product reached

TABLE I

Inhibition of ØX174 RF → RF Synthesis by a dUTPase Defect

Enzyme Preparation	cisA Protein	Nucleotide Incorporation (pmol)
dut$^+$	−	9
dut$^-$	−	6
dut$^+$	+	216
dut$^-$	+	27

ØX RF → RF synthesis was performed as described by Eisenberg *et al.* (5) using enzyme preparations (Fraction II) derived from *dut*$^-$ and *dut*$^+$ strains with purified *cis*A protein added as indicated.

a maximum within a few minutes after the start of the reaction and then rapidly declined upon further incubation. Thus, uracil, once incorporated, is rapidly excised.

ØX RF Replication. Supercoiled ØX RF can undergo replication in partially purified extracts of *E. coli* fortified with the ØX174-coded *cis*A protein (6, 7). The extent of this reaction was decreased by nearly 10-fold in preparations derived from a *dut*$^-$ mutant as compared with the wild type strain (Table I). Inhibition of ØX RF replication, as a consequence of the incorporation of uracil followed by its excision, is plausibly accounted for by the fact that the *cis*A protein initiates ØX RF replication by the introduction of a nick specifically in supercoiled DNA (11, 12). Inasmuch as multiple rounds of replication occur on a single template (7, 12), introduction of a nick after the first round of replication would be expected to render it nonfunctional for further replication.

Conversion of ØX RF to Single Stranded Progeny DNA. Four enzymes are required for the conversion of ØX RF to single stranded progeny molecules: the *cis*A protein, DNA polymerase III holoenzyme, the *rep* protein and DNA binding protein, all of which are nearly homogeneous and free of dUTPase (12, 13). In contrast to the single stranded circular DNA isolated from the virion, the single stranded ØX174 DNA synthesized by these enzymes *in vitro* is relatively inefficient as a template in the SS → RF reaction (7). The latter requires at least ten proteins, several of which have not yet been purified. Thus, any incorporation of uracil into the single stranded product during RF → SS synthesis in the absence of dUTPase would result in its cleavage upon exposure to the uracil N-glycosidase, and apurinic endonuclease that might

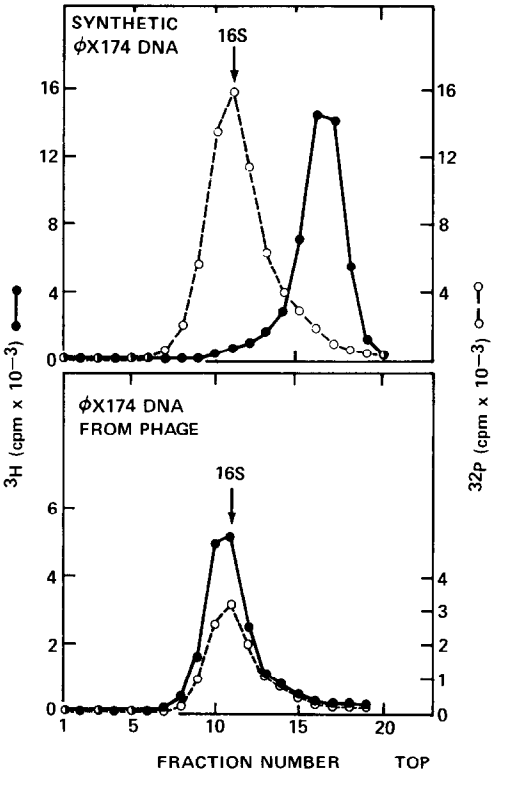

Figure 4: Presence of uracil in enzymatically synthesized ϕX174 SS DNA. (Top) The reaction mixture (10 μl) contained 0.01 mM (in nucleotides) synthetic ^{3}H-labeled ϕX SS DNA (464 cpm/pmol), 50 mM Tris-HCl (pH 7.5), 2 mM EDTA and 1.2 units/ml uracil N-glycosidase. (Bottom) The reaction mixture (100 μl contained 0.025 mM ^{3}H-labeled single stranded DNA (3300 cpm/nmol)) isolated from ϕX174; the remaining components were the same as above. After incubation at 37° for 30 min, the mixtures were chilled to 0°, and 100 μl of a solution composed of 0.8 M NaCl, 0.2 N NaOH and 2 mM EDTA were added together with ^{32}P-labeled single stranded DNA isolated from ϕX174, which served as a sedimentation marker, and 25 μg of calf thymus DNA as carrier. The mixture was sedimented in a 5-20% alkaline sucrose density gradient in a Beckman L260B centrifuge with an SW50 rotor at 53,000 rpm at 15° for 3.5 hr. Sedimentation of the synthetic ^{3}H-labeled ϕX SS DNA without uracil N-glycosidase treatment showed a single species with a sedimentation coefficient of 16S.

contaminate the proteins required for the SS → RF conversion. In fact, treatment with purified uracil N-glycosidase of the single stranded DNA synthesized in vitro resulted in its degradation to 8S fragments. In contrast, the single stranded DNA isolated from the phage was completely insensitive to the action of uracil N-glycosidase (Fig. 4).

Shlomai and Kornberg have, recently, found that addition of purified dUTPase to the ØX RF → SS reaction mixture results in the synthesis of a product that serves as an effective template for SS → RF conversion (13).

DISCUSSION

Incorporation of uracil into DNA leads in vitro, as it does in vivo, to extensive cleavage of the product by a post-replication excision-repair system that consists of uracil N-glycosidase and an apurinic endonuclease. Even in the absence of the latter, the conditions of alkaline sucrose density gradient sedimentation used in the analysis of newly synthesized DNA are sufficient to cause phosphodiester bond cleavage at sites of uracil excision (14).

Shlomai and Kornberg have found that the K_m of DNA polymerase III holoenzyme for dUTP and dTTP are nearly the same (13). Hence, in the absence of dUTPase, contamination of the deoxynucleoside triphosphates with dUTP at a level of 0.1% is sufficient to generate a cleavage site at 1000-nucleotide intervals. The origin of the endogenous dUTP is not known. However, contamination of dCTP with dUTP either as a result of dCTP deaminase (15) or chemical deamination are both reasonable possibilities (16).

Although we have detected cleavage of newly synthesized DNA in vitro in enzyme preparations with reduced levels of dUTPase, resulting from a dut mutation, it is clear that any fractionation procedure applied to wild type extracts that results in the removal of dUTPase while at the same time retaining uracil N-glycosidase will produce similar effects. Thus, the single stranded DNA synthesized from ØX RF by a group of highly purified proteins (DNA polymerase III holoenzyme, DNA binding protein, the cisA protein and rep protein) is an intact circle. However, exposure of this product to the ten proteins required for SS → RF synthesis, several of which have not been purified, results in prompt fragmentation, presumably at sites within the molecule at which uracil had been incorporated in the course of its synthesis from RFI.

A reasonable conclusion that may be drawn from these studies is that small DNA fragments that are generated during DNA synthesis both in vitro and in vivo need not necessarily represent the products of de novo initiation events. Furthermore, enzymes, for example, dUTPase, that appear to be required for DNA replication in vitro may not be directly involved in DNA replication in vivo.

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