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Excision Repair of Uracil Incorporated in DNA as a Result of a Defect in dUTPase

BIK-KWOON TYE† AND I. R. LEHMAN

Department of Biochemistry
Stanford University School of Medicine
Stanford, Calif. 94305, U.S.A.

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Mutants of *Escherichia coli* that are severely defective in the enzyme dUTPase (*dut*) accumulate short (4 to 5 S) Okazaki fragments following brief pulses with [^{3}H]thymidine. The transient appearance of DNA fragments in these mutants is plausibly explained by the misincorporation of uracil in DNA as a result of an increase in available dUTP, followed by its rapid excision and repair. The evidence in support of this interpretation is the following: (1) accumulation of short DNA fragments can be partially suppressed by a mutation in dCTP deaminase, presumably by decreasing the intracellular level of dUTP relative to dTTP; (2) accumulation of the short DNA fragments can be almost completely suppressed by a mutation in uracil *N*-glycosidase, probably by preventing the introduction of nicks at the sites of uracil incorporation; (3) introduction of DNA polymerase I or DNA ligase mutations into dUTPase-defective strains results in the persistence of the 4 to 5 S fragments and rapid cessation of DNA synthesis. Uracil *N*-glycosidase, DNA polymerase I and DNA ligase must therefore be involved in the excision repair of uracil-containing DNA.

1. Introduction

When mutants of *Escherichia coli* defective in the enzyme dUTPase (*dut*) are exposed to brief pulses with [^{3}H]thymidine, labeled DNA fragments appear that are rapidly incorporated into high molecular weight DNA, and hence resemble the now classic Okazaki fragments. The size of the labeled fragments and the extent to which they persist depend on the severity of the dUTPase defect (Tye *et al.*, 1977). In the experiments reported here, we have examined the effects of a defect in DNA polymerase I and in DNA ligase on the behavior of a *dut* mutant in which the labeled fragments are abnormally small, 4 to 5 S (the Sof phenotype (Konrad & Lehman, 1975)). We have found that introduction of a non-lethal *polA* or *lig* mutation into this strain results in (1) temperature-sensitive conditional lethality, (2) inability of the small pulse-labeled fragments to be joined, and (3) rapid shut-off of DNA replication. DNA polymerase I and DNA ligase must therefore be involved in the repair of uracil-induced damage to DNA.

We have also observed that introduction of a mutation in uracil *N*-glycosidase (*ung*) into the *dut*$^-$ strain completely suppresses the abnormal accumulation of the

† Present address: Section of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, N.Y. 14853, U.S.A.

small fragments. Hence, this enzyme is also very likely involved in the post-replication excision repair of uracil-induced damage to DNA. Finally, we have found that elimination of dCTP deaminase, an enzyme that catalyzes the deamination of dCTP to generate dUTP, diminishes the extent to which small pulse-labeled fragments accumulate. These findings provide strong support for the idea suggested earlier (Tye *et al.*, 1977), that a decrease in dUTPase results in an increased incorporation of uracil into DNA; rapid removal of the uracil by an excision repair process involving uracil *N*-glycosidase, DNA polymerase I and DNA ligase then leads to the transient appearance of the small DNA fragments.

2. Materials and Methods

(a) *Media*

The liquid media used were M63 minimal salts medium, TY medium, and Tryptone broth prepared as described by Miller (1972). Solid media were: M63 medium containing 1·5% agar, TY medium containing 1·5% agar, Tryptone containing 1·5% agar, and TY-MMS agar, which is composed of TY medium containing 1·5% agar and 0·04% methylmethane sulfonate. H-top agar was prepared according to Gottesman & Beckwith (1969).

(b) *Strains and bacteriological methods*

The *E. coli* K12 strains used are given in Table 1.

We have adopted the designations *dut*-11, *dut*-12 and *dut*-13 for the *sof*-1, *sof*-2 and *sof*-3 mutations, respectively, in conformity with accepted nomenclature for the dUTPase genetic locus (Tye *et al.*, 1977).

Bacterial matings were carried out according to Miller *et al.* (1968), and transductions with P1*vir* phage were performed as described by Signer (1966), except that transductants were selected on plates supplemented with 5 mM-sodium citrate.

P1*vir* stocks were made by the following method. A culture was grown to a titer of about 2×10^8/ml, made 5 mM in $CaCl_2$, and incubated for 5 min at 37°C. A portion (1 ml) of culture was mixed with 10^5 P1*vir* phage, incubated for 20 min, then plated in 2·5 ml of H-top agar. Phage were harvested after 12 h.

his$^-$ deletion mutants were isolated by P2 prophage eduction as described by Miller (1972). Phage P2 *lgcc* was a gift from Dr Richard Calendar.

(c) *Isolation of pulse-labeled DNA*

The following procedure, modified from Okazaki *et al.* (1968), was used for isolation of DNA pulse-labeled with [^{3}H]thymidine. Cultures were grown to an A_{595} of about 0·5 at 30°C in M63 medium supplemented with 0·2% glucose, 0·5% Casamino acids, 10 μg thiamine/ml, and 2 μg thymine/ml. A portion (6 ml) of culture was removed and shaken for 5 min at the temperature (30°C or 44°C) at which it was to be pulsed. Then 100 μl of [^{3}H]thymidine (20 Ci/mmol 1 mCi/ml; New England Nuclear) was added. The pulse was terminated by addition of an equal volume of 75% ethanol, 2% phenol, 10 mM-sodium acetate, 2 mM-EDTA. The resulting suspension was centrifuged and the pellet resuspended in 0·6 ml of 0·2 M-NaOH, 10 mM-EDTA. ^{32}P-labeled φX174 (a gift of Dr Schlomo Eisenberg of this Department) was added as a sedimentation marker, and the mixture was incubated at 37°C for 1 h.

(d) *Alkaline sucrose density-gradient centrifugation*

Alkaline sucrose gradients (5% to 20%) were run in a Beckman L265B ultracentrifuge using an SW41 Ti rotor for 14 h at 40,000 revs/min at 5°C in solutions containing 0·8 M-NaCl, 0·2 M-NaOH and 2 mM-EDTA in addition to the sucrose. At the end of the run, 0·4-ml fractions were collected from the bottom of the tube. Salmon sperm DNA (0·1 ml of 0·4 mM-nucleotide) was added to each fraction, followed by 0·6 ml of cold 10% trichloroacetic acid. Precipitates were collected on Whatman GF/C glass filters and washed with 4 portions (5 ml each) of cold 1·0 M-HCl and 2 portions (3 ml each) of ethanol.

TABLE 1

E. coli *K12 strains used in construction of double mutants*

Strain	Genotype	Reference or construction
MM383	W3110 (*E. coli* C strain) *pol*A12 *rha*$^-$ *thy*$^-$ *lac*$^-$ *str*^{r}	Monk & Kinross (1972)
KS243	Hfr KL16 *thi*$^-$ *pts*-140	Strain CHEp40 from W. Epstein
SP1	F$^-$ *str*A *lop*-11	M. Gellert
58M4	Hfr Cavali arbutin$^+$ *pyr*E$^-$ *met*$^-$ *str*^{s}	
KS474	F$^-$ *met*B$^-$ *thi*$^-$ *lac* MS286 ϕ80dII *lac* BK1 *str*^{r}	Konrad & Lehman (1975)
RS5087	F$^-$ *met*B$^-$ *thi*$^-$ *lac* MS286 ϕ80dII *lac* BK1 *dut*-11 *str*^{r}	Konrad & Lehman (1975)
RS5090	F$^-$ *lac* MS286 ϕ80dII *lac* BK1 *dut*-11 *pts*-140 *str*^{r}	Mating between KS243 and RS5087 from E. B. Konrad
BKT102	F$^-$ *lac* MS286 ϕ80dII *lac* BK1 *dut*-11 *lop*-11 *str*^{r}	RS5090 transduced with P1*vir* grown on SP1; this work
BKT103	F$^-$ *pol*A12 *rha*$^-$ *str*^{r} *thy*A$^-$ *lac* Y14 *pyr*E$^-$ arbutin$^+$	Mating between 58M4 and MM383; this work
BKT106	F$^-$ *lac* MS286 ϕ80dII *lac* BK1 *dut*-11 *lig*-4 *str*^{r}	RS5090 transduced with P1*vir* grown on N1626; this work
BKT108	F$^-$ *lac*Y$^-$ *str*^{r} *thy*A$^-$ *rha*$^-$ *dut*-11 *pol*A12	BKT103 transduced with P1*vir* grown on RS5087; this work
N1626	F$^-$ *str*^{r} *lig*-4 *pts*$^+$	M. Gellert
BD1153	F$^-$ *thi*-1 *arg*H1 *lac*Y1 *gal*-6 *mal*A1 *xyl*-7 *rha*-6 *ara*-13 *str*A9 *sup*E-44 λ^R *ung*-1	H. Warner & B. Duncan
BD1154	F$^-$ *thi*-1 *arg*H1 *lac*Y1 *gal*-6 *mal*A1 *xyl*-7 *rha*-6 *ara*-13 *str*A9 *sup*E-44 λ^R	H. Warner & B. Duncan
BD1156	F$^-$ *thi*-1 *arg*H1 *lac*Y1 *gal*-6 *mal*A1 *xyl*-7 *rha*-6 *ara*-13 *str*A9 *sup*E-44 λ^R *dut*-1	H. Warner & B. Duncan
BD1157	F$^-$ *thi*-1 *arg*H1 *lac*Y1 *gal*-6 *mal*A1 *xyl*-7 *rha*-6 *ara*-13 *str*A9 *sup*E-44 λ^R *dut*-1 *ung*-1	H. Warner & B. Duncan

(c) *Assay of dCTP deaminase*

Cultures (400 ml) were grown in M63 medium supplemented with 0·2% glucose, 0·5% Casamino acids, 10 μg thiamine/ml and 2 μg thymine/ml to $A_{595} = 0{\cdot}75$. They were then harvested, washed with 0·15 M-NaCl, resuspended in 1 ml of 50 mM-potassium phosphate (pH 6·8), 2 mM-EDTA, 1 mM-dithiothreitol, and sonicated for 1 min. The extracts were centrifuged, and the supernatant fluid treated with 0·25 vol. 15% streptomycin sulfate at 0°C. After 30 min, the suspensions were centrifuged. The supernatant fluid was dialyzed for 2 to 4 h against 500 vol. of the sonication buffer and assayed directly (Beck *et al.*, 1977).

The reaction mixture contained in 20 μl, 50 mM-potassium phosphate (pH 6·8), 1 mM-dithiothreitol, 1 mM-[^{3}H]dCTP (spec. act. 24·1 Ci/mmol), 5 mM-$MgCl_2$ and extract. Incubation was at 37°C for 20 min, then 1 μl samples were withdrawn and applied directly to PEI-cellulose thin-layer plates together with 10 nmol each of dCTP, dCDP, dCMP, dUTP, dUDP and dUMP as markers. The plates were washed for 10 min in absolute methanol, dried, and developed stepwise in 1 dimension. The solvents used were the following: (1) absolute methanol to the starting line; (2) 1 M-formic acid to 1 cm above the starting line; (3) 1 M-formic acid, 0·25 M-LiCl to 3 cm above the starting line; (4) 1 M-formic acid, 1·25 M-LiCl to 12 cm above the starting line. After drying with hot air, the spots corresponding to dCTP, dCDP, dCMP, dUTP, dUDP and dUMP were identified under ultraviolet light, cut out, and placed in counting vials together with 0·2 ml of 1 M-NH_4HCO_3 to elute the nucleotides from the PEI-cellulose. After 20 min at room temperature, 8 ml of a Triton X100/toluene scintillation fluid was added to each vial and the radioactivity determined in a Beckman LS-230 scintillation spectrometer.

From the radioactivity in each of the nucleotides, the amount of conversion of dCTP to dUTP and dUMP (the latter resulting from the action of dUTPase) was calculated. The assay was linear in the range of 0·1 to 1·0 nmol of dCTP converted to dUTP (+dUMP).

(f) *Histidine complementation tests*

The following strains of *Salmonella typhimurium* (gifts from Dr John Roth and John Scott) were used in *his* complementation tests: (1) derivatives of LT2 *trp*A8 *pur*E801 *his*612 (BHAFIE deletion) carrying *E. coli* F′ *his*bD2377, F′ *his*abD2382; (2) derivatives of LT2 *ser*821 *arg*501 *his*721 (DCBHAFIE deletion) carrying *E. coli* F′ *his*$^+$, F′ *his* C2385, *F′ his*a(b)cdB2405, F′ *his*A2406, F′ *his*a(b)E2414, F′ *his*a(b)cdB2415; (3) LT2 F$^-$*his* DG. (The lower case letters denote deficiencies of the mutant with respect to intragenic complementation groups.)

his$^-$ auxotrophs carrying point mutations should be complemented by *Salmonella* strains carrying F′ factors that are mutant in any of the *his* structural genes other than that in which the point mutation occurs. In contrast, deletions that extend through several genes are not complemented by F′ factors that carry *his*$^-$ mutations in any of the *his* structural genes included in the deletion.

Complementation tests of *his*$^-$ auxotrophs by *Salmonella* carrying *E. coli* F′ *his* episomes involved streaking out the test strain and *Salmonella* F′ *his* donor strains on minimal plates and scoring for growth where the two strains overlap. Neither the donor nor recipient strain are able to grow on minimal medium, since the donor is auxotrophic for several nutritional requirements and the recipient is auxotrophic for histidine.

3. Results

(a) *The* dut-*11* pol*A12 double mutant*

The labeling patterns observed after subjecting strains with either the *pol*A12 or *dut*-11 mutation to ten-second pulses with [^{3}H]thymidine at 44°C are shown in Figure 1. The fragments in the *dut*-11 strain were smaller than those in the *pol*A12 mutant (4·4 S *versus* 8·8 S). In other *dut* mutants, with levels of dUTPase higher than that in *dut*-11 (4% of wild type), the pulse-labeled fragments had average sedimentation coefficients close to that seen in the *pol*A mutant (Tye *et al.*, 1977).

Under all conditions examined, survival of the double mutant *E. coli dut*-11 *pol*A12 at 43°C was significantly less than the *dut*-11 or *pol*A12 strains from which it was constructed (Table 2). In addition, the double mutant, unlike either parent, was abnormally sensitive to methylmethane sulfonate even at 30°C.

Upon shift of a culture of the double mutant from 30°C to 44°C, DNA synthesis ceased within ten minutes (Fig. 2). Thus, combination of the *dut*-11 and *pol*A12 mutations results in the inability of the double mutant to grow at 44°C and blocks DNA synthesis at this temperature.

The defect in DNA replication in the *dut*-11 *pol*A12 double mutant was further examined in pulse-labeling experiments. A culture of the double mutant was grown at 30°C, then shifted to 44°C and pulsed with [^{3}H]thymidine for increasing period of time. In contrast to the *dut*-11 mutant, the 4 to 5 S fragments that appeared in the double mutant persisted for pulse periods ranging from ten seconds to five minutes (Fig. 3). Thus, at 44°C, the *pol*A12 mutation appears to block conversion of the 4 to 5 S DNA fragments to high molecular weight DNA. In contrast, the sedimentation profile of DNA isolated from the *dut*-11 *pol*A12 double mutant after pulsing with [^{3}H]thymidine at 30°C was similar to that of the *dut*-11 mutant at this temperature, i.e. [^{3}H]thymidine was incorporated into DNA of increasing molecular weight as length of the pulse increased.

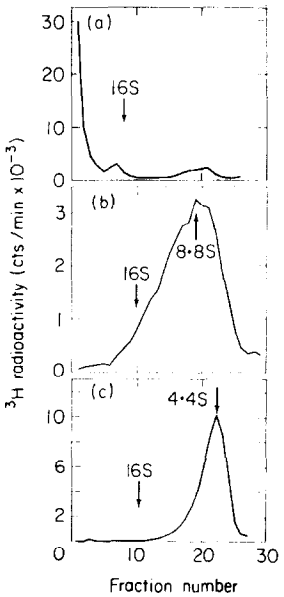

FIG. 1. Sedimentation profiles of DNA in alkaline sucrose density-gradients after [^{3}H]thymidine pulse-labeling of wild type and *pol*A12 and *dut*-11 mutants of *E. coli*.

Cultures (6 ml) were grown in supplemented M63 medium at 33°C to A_{595} = 0·5, then shifted to 44°C for 5 min and pulsed with 100 μl of [^{3}H]thymidine (spec. act. 20 mCi/μmol, 1 mCi/ml) for 10 s. The cultures were lysed and the DNA sedimented in a 5% to 20% alkaline sucrose density-gradient as described in Materials and Methods. (a) Wild type (KS474); (b) *pol*A12 (MM383); (c) *dut*-11 (RS5087).

TABLE 2

Effect of lig *and* pol*A mutations on the survival of the* dut-*11 mutant at 43°C*

Strain	TY-MMS	Medium TY Survival at 43°C (%)	Tryptone	M63
*lig*4	100	100	100	100
*pol*A12	$1{\cdot}5\times10^{-3}$	1·0†	23	100‡
*lig*4 *dut*-11	10^{-2}	80	62	2
dut-11	100	57	85	100
dut-11 *pts*	68	100	60	100
*pol*A12 *dut*-11	$<10^{-5}$	$1{\cdot}4\times10^{-3}$	0·1	2‡

Cells were grown to A_{595} = 0·5 at 30°C in M63 medium supplemented with 0·2% glucose, 0·5% Casamino acids, 10 μg thiamine/ml and 2 μg thymine/ml and then plated as indicated at 30°C and 43°C.

† The *pol*A12 mutant used in these experiments, in contrast to the strain isolated initially (Monk & Kinross, 1972), is somewhat temperature sensitive.

‡ The plating efficiency (number of colonies relative to A_{595}) was low even at 30°C.

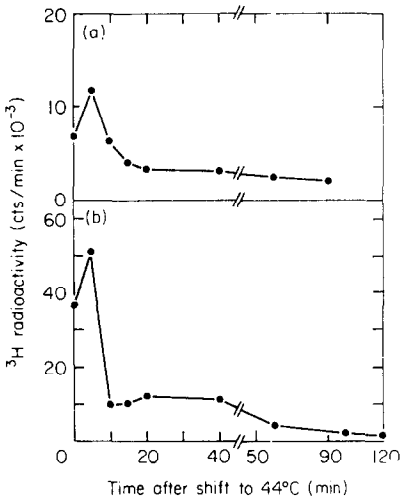

FIG. 2. Rate of incorporation of [^{3}H]thymidine in *dut*-11 *pol*A12 and *dut*-11 *lig*-4 double mutants at 44°C.

Cultures (10 ml) were grown in supplemented M63 medium at 30°C to A_{595} = 0·5, then shifted to 44°C. Portions (0·5 ml) were pulsed with 10 μl of [^{3}H]thymidine (20 mCi/μmol, 1 mCi/ml) for 30 s, then 0·2 ml of ice-cold 17% trichloroacetic acid containing 2·5 mg unlabeled thymidine/ml was added. Acid-insoluble radioactivity was measured as described in Materials and Methods. (a) *dut*-11 *pol*A12; (b) *dut*-11 *lig*-4.

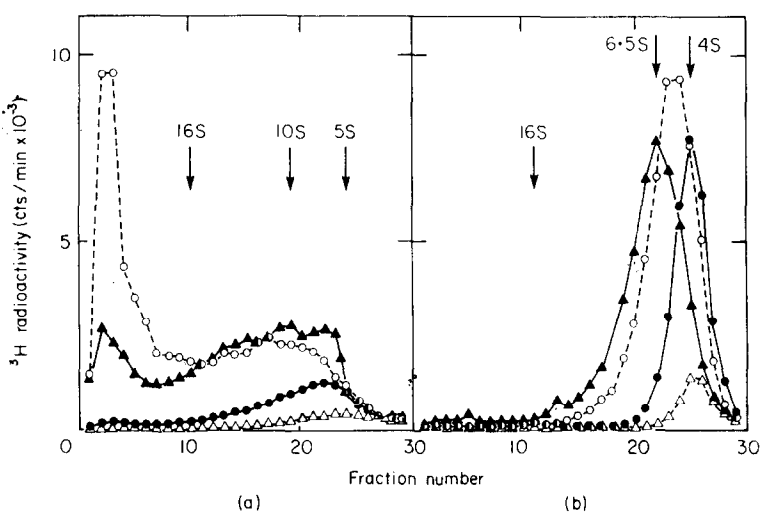

FIG. 3. Sedimentation profiles of DNA in alkaline sucrose density-gradients after [^{3}H]thymidine pulse-labeling of the *dut*-11 *pol*A12 double mutant.

Cultures were grown at 30°C in supplemented M63 medium to A_{595} = 0·5, then (a) pulsed with [^{3}H]thymidine at 30°C as described in the legend to Fig. 1, for 10 s (—△—△—), 30 s (—●—●—), 1 min (—▲—▲—), and 2 min (--○--○--); (b) shifted to 44°C for 5 min before being pulsed with [^{3}H]thymidine for 10 s (—△—△—), 30 s (—●—●—), 2 min (--○--○--) and 5 min (—△—△—). The cultures were lysed and the DNA sedimented in a 5% to 20% alkaline sucrose density-gradient as described in Materials and Methods.

(b) *Conversion of 4 to 5 S to 8 to 10 S DNA fragments in the* dut-*11* pol*A12 double mutant*

To examine the fate of the 4 to 5 S fragments upon suppression of the *pol*A12 mutation, pulse-chase experiments were done in which the *dut*-11 *pol*A12 double mutant was pulsed with [^{3}H]thymidine for one minute at 44°C, shifted to 30°C and then treated with a large excess of cold thymidine (100 μg/ml) for 0·5, 1, 2 and 5 minutes. As shown in Figure 4, there was a significant increase in the sedimentation coefficient of the pulse-labeled DNA with increasing time of exposure to unlabeled thymidine. Furthermore, label appeared in 8 to 10 S material before being incorporated into large molecular weight DNA, suggesting that the 4 to 5 S fragments might be precursors of the 8 to 10 S Okazaki fragments.

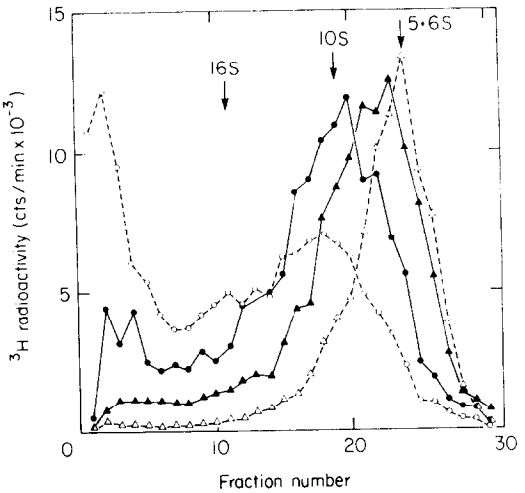

FIG. 4. Sedimentation profiles of DNA in alkaline sucrose density-gradients following pulse-chase of the *dut*-11 *pol*A12 double mutant.

Cultures were grown at 30°C in supplemented M63 medium to an A_{595} = 0·5, then shifted to 44°C for 5 min, and pulsed with [^{3}H]thymidine for 1 min, as described in the legend to Fig. 1. The cultures were then cooled to 30°C and an excess (100 μg/ml) of unlabeled thymidine was added for 0 min (--△--△--), 1 min (—▲—▲—), 2 min (—●—●—), 5 min (--○--○--). The cultures were lysed and the DNA sedimented in a 5% to 20% alkaline sucrose density-gradient as described in Materials and Methods.

(c) *The* dut-*11* lig-*4 double mutant*

Like the *dut*-11 *pol*A12 double mutant, there was a rapid cessation of DNA synthesis on shift of the *dut*-11 *lig*-4 mutant from 30°C to 44°C (Fig. 2(b)). Again, like the *dut*-11 *pol*A12 double mutant, [^{3}H]thymidine was incorporated almost exclusively into 4 to 6 S DNA fragments, regardless of the length of the pulse period (Fig. 5).

(d) *The* dut-*11* lop-*11 double mutant*

Inasmuch as DNA ligase appears to be required for the joining of the 4 to 5 S fragments, the effect of the mutation *lop*-11 (Gellert & Bullock, 1970), which leads to a fivefold increase in the level of this enzyme in extracts, was examined. Cultures of *E. coli dut*-11 *lop*-11 were pulsed with [^{3}H]thymidine at 43°C for ten seconds. As shown in Figure 6, most of the labeled DNA sedimented at 4 to 5 S. However, unlike the

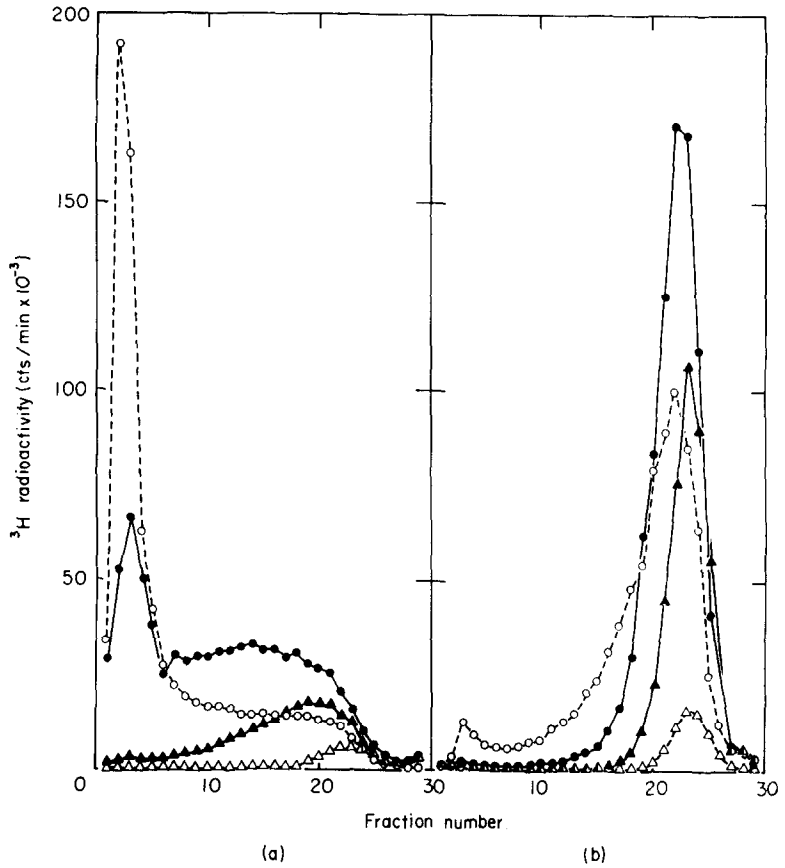

FIG. 5. Sedimentation profiles of DNA in alkaline sucrose density-gradients after [^{3}H]thymidine pulse-labeling of the *dut*-11 *lig*-4 double mutant.

Cultures were grown at 30°C in supplemented M63 medium to A_{595} = 0·5, then (a) pulsed with [^{3}H]thymidine at 30°C as described in the legend to Fig. 1, for 10 s (—△—△—), 30 s (—▲—▲—), 1 min (—●—●—), and 2 min (--○--○--); (b) shifted to 44°C for 5 min before being pulsed with [^{3}H]thymidine for 10 s (—△—△—), 30 s (—▲—▲—), 2 min (—●—●—), and 5 min (--○--○--). The cultures were lysed and the DNA sedimented in a 5% to 20% alkaline sucrose density gradient as described in Materials and Methods.

dut-11 mutant, there was a shoulder with a sedimentation coefficient of 8 to 10 S. Thus, an increase in the level of DNA ligase can overcome partially the *dut* mutation, presumably by increasing the rate of conversion of 4 to 5 S to 8 to 10 S DNA fragments.

(e) *Supression of Sof phenotype by a mutation in uracil* N-*glycosidase*

Uracil *N*-glycosidase cleaves the *N*-glycosidic bond linking uracil to deoxyribose in DNA (Lindahl, 1974) and may, therefore, be involved in the removal of uracil residues in DNA. Mutants are known that are defective in the uracil *N*-glycosidase (*ung*) (Duncan & Warner, 1976); however, they have no obvious phenotype. To determine whether a defect in uracil *N*-glycosidase can decrease the level of 4 to 5 S fragments that appear in the *dut*-11 mutant, the double mutant *dut*-1 *ung*-1 was examined. When cultures of the double mutant were pulsed with [^{3}H]thymidine for ten seconds at 30°C, a sedimentation profile similar to that of the wild type was

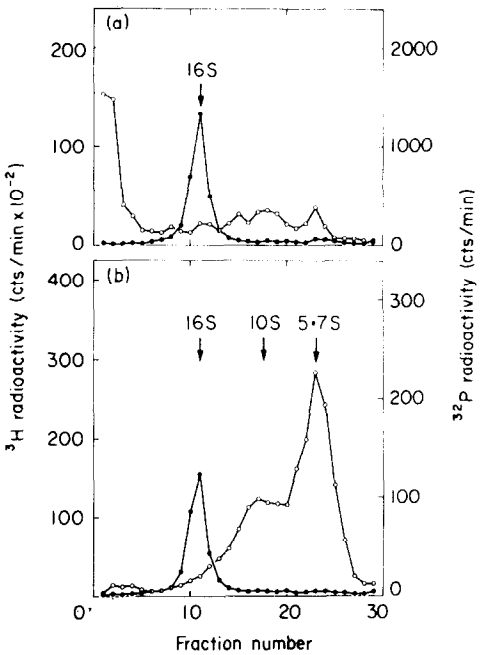

FIG. 6. Sedimentation profiles of DNA in alkaline sucrose density-gradients after [^{3}H]thymidine pulse-labeling of the *dut*-11 *lop*-11 double mutant.

Cultures were grown in supplemented M63 medium at 30°C to $A_{595} = 0{\cdot}5$, then shifted to 43°C for 5 min, and pulsed with [^{3}H]thymidine for 10 s as described in the legend to Fig. 1. ^{32}P-labeled ϕX174 DNA served as a sedimentation marker. (a) *lop*-11; (b) *dut*-11 *lop*-11. The cultures were lysed and the DNA sedimented in a 5% to 20% alkaline sucrose density-gradient as described in Materials and Methods.

observed (Fig. 7). Thus, an *ung*$^-$ mutation suppresses the Sof phenotype, presumably by preventing cleavage of phosphodiester bonds at apyrimidinic acid sites generated by the action of *N*-glycosidase†.

(f) *Partial suppression of the Sof phenotype by loss of dCTP deaminase activity*

As noted above, the *dut*-11 *pol*A12 double mutant, in contrast to either of the single mutants, is temperature sensitive for growth (Table 2). A group of temperature-resistant revertants selected at 43°C on TY plates remained sensitive to methylmethane sulfonate and thus retained the *pol*A12 mutation. However, almost all (63 out of 64) of the revertants were auxotrophic for histidine. Mutants defective in DNA polymerase I are known to accumulate auxotrophs (Berg, 1971) and to generate deletions at a high frequency (Conkell & Yanofsky, 1970). Since the genetic locus for dCTP deaminase (*dcd*) is very near the *his* region, it was likely that the *his*$^-$ revertants contained deletions in the *his* region extending through the *dcd* locus. Three of the

† Growth of the *dut*-1 *ung*-1 double mutant in the presence of [6-^{3}H]uridine results in substantial replacement of thymine by uracil in the DNA of these cells, but not in wild type or *ung*-1 cells. (H. B. Warner & B. Duncan, personal communication.)

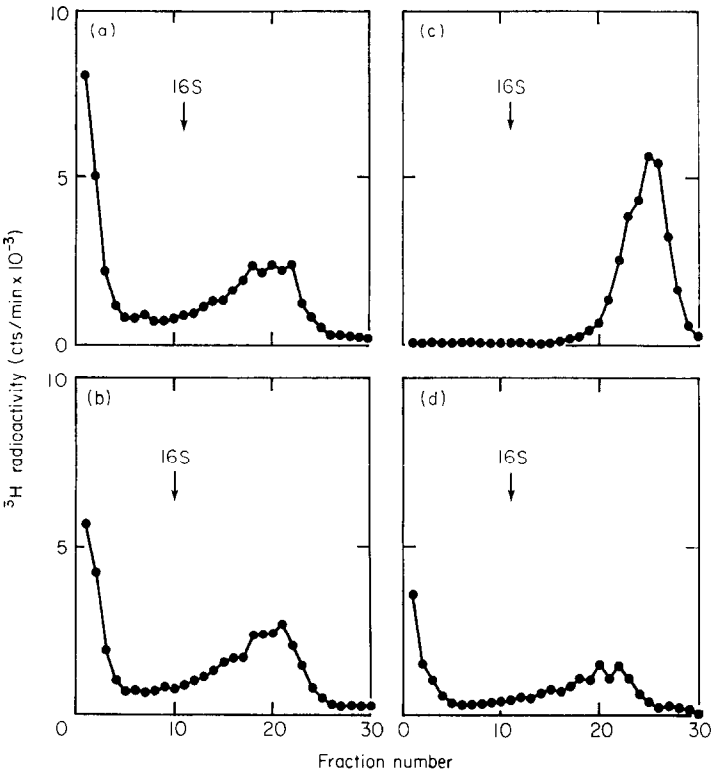

FIG. 7. Sedimentation profiles of DNA in alkaline sucrose density-gradients after [^{3}H]thymidine pulse-labeling of wild type strain, and *ung*-1, *dut*-1 and *dut*-1 *ung*-1 mutants of *E. coli*.

Cultures were grown in supplemented M63 medium at 30°C to A_{595} = 0·5, then pulse-labeled with [^{3}H]thymidine at 30°C for 10 s as described in the legend to Fig. 1. (a) *ung*-1; (b) wild type; (c) *dut*-1; (d) *dut*-1 *ung*-1.

TABLE 3

Complementation in his$^-$ *revertants of BKT108* (dut-*11* pol*A12*) *with* Salmonella *strains carrying an* E. coli *F′* his *factor*

	LT-2 F$^-$his ΔG	BKT108R1	BKT108R2	BKT108R3
F′ *his*$^+$	+	+	+	+
F′ *his* D2377	+	−	−	−
F′ *his* D2382	+	−	−	−
F′ *his* C2385	+	−	−	−
F′ *his* B2405	+	−	−	−
F′ *his* A2406	+	−	−	−
F′ *his* E2414	+	−	−	−
F′ *his* B2415	+	−	−	−

his$^-$ auxotrophs were tested for their ability to complement a series of F′ factors (carrying heterologous *his* genes from *E. coli*), each of which was mutant in one of the *his* structural genes (see Materials and Methods). A *Salmonella* strain which has a *his* deletion in the *his*G gene was used as a control.

TABLE 4

dCTP deaminase activity of E. coli dut-*11* pol*A12 pseudorevertants*

Strain	Genotype	dCTP deaminase (% of wild type)
Wild type		100
RS5087	*dut*-11	94
BKT108	*dut*-11 *pol*A12	106
BKT108R1	*dut*-11 *pol*A12 ∇ *his*	9
BKT108R2	*dut*-11 *pol*A12 ∇ *his*	4
BKT108R3	*dut*-11 *pol*A12 ∇ *his*	2

Values are relative to that of wild type extracts (0·3 nmol of dCTP deaminated/min per mg protein). Enzyme assays were performed as described in Materials and Methods.

his⁻ mutants were subjected to complementation tests after mating with *Salmonella* strains, each with an *E. coli* F′ episome containing the *his* operon mutant in a different gene of the operon. All three of the mutants bore deletions of the *his* region (Table 3) and lacked dCTP deaminase activity (Table 4). Thus, introduction of a dCTP deaminase deletion restores the viability of the double mutant, *dut*-11 *pol*A12, at 43°C. It also suppresses the Sof phenotype. The alkaline sucrose sedimentation profile of the pulse-labeled DNA of the triple mutant *dut*-11 *pol*A12 ∇ *dcd* showed predominantly 8 to 10 S fragments, unlike the 4 to 5 S fragments seen in the *dut*-11 *pol*A12 double mutant (Fig. 8). However, the Sof phenotype was only partially suppressed by the *dcd* deletion. When the *dut*-11 ∇ *dcd* double mutant was constructed, either by

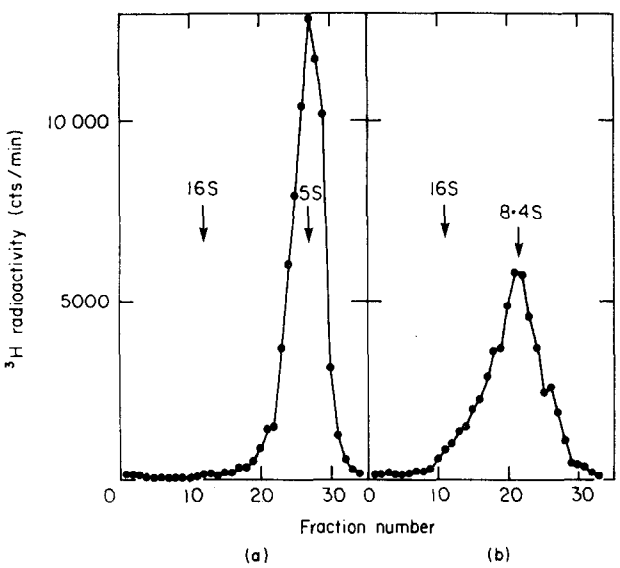

FIG. 8. Sedimentation profiles of DNA in alkaline sucrose density-gradients after [³H]thymidine pulse-labeling of the *dut*-11 *pol*A12 double mutant and *dut*-11 *pol*A12 bearing a dCTP deaminase (*dcd*) deletion.

Cultures were grown in supplemented M63 medium at 30°C to $A_{595} = 0{\cdot}5$ then shifted to 43°C for 5 min and pulsed with [³H]thymidine at 43°C for 30 s as described in the legend to Fig. 1. (a) *dut*-11 *pol*A12 (BKT108); (b) *dut*-11 *pol*A12 ∇ *his* ∇ *dcd* (BKT108R1).

reversion of the *dut*-11 *pol*A12 ∇ *dcd* strain to methylmethane sulfonate resistance, or by introduction of a *dcd* deletion into the *dut*-11 strain by education of phage P2, there was little or no suppression of the Sof phenotype as judged by alkaline sucrose sedimentation of the pulse-labeled DNA. However, the *dut*-11 ∇ *dcd* P2 eductant was no longer hyper rec (Konrad, 1977).

4. Discussion

DNA polymerase I, DNA ligase and uracil *N*-glycosidase are all required for the excision repair of uracil incorporated in DNA as a result of a defective dUTPase. A plausible sequence to account for this process is shown in Figure 9. Uracil *N*-glycosidase specifically cleaves the *N*-glycosidic bond linking uracil to deoxyribose. Endonuclease II (Friedberg *et al.*, 1969; Weiss, 1976) or the apurinic endonuclease (Verley & Rassart, 1975) could then cleave the phosphodiester bond at the apyrimidinic acid site created by excision of uracil. The 5′ → 3′ exonuclease associated with DNA polymerase I may remove the deoxyribose phosphate moiety remaining after the excision, and the polymerase function could fill in the gap that remains. Finally, DNA ligase could seal the nick and complete the repair process.

The abrupt halt in DNA synthesis on shift of the *dut*-11 *pol*A12 and *lig*-4 *dut*-11 double mutants to restrictive temperature suggests that persistence of uracil-induced nicks and/or gaps as a consequence of a defective DNA polymerase I or DNA ligase may very quickly destroy the replication fork.

Synthesis of dUTP occurs by two routes: deamination of dCTP by dCTP deaminase (Beck *et al.*, 1975), and reduction of UDP to dUDP by ribonucleoside diphosphate reductase (Reichard, 1967) followed by its conversion to the triphosphate by nucleoside diphosphate kinase. If the transient accumulation of DNA fragments in the *dut* mutants is a consequence of a defect in dUTPase, leading to an increase in the intracellular level of dUTP, then a decrease in dUTP concentration, resulting from inhibition of one or both of the pathways, might alleviate the dUTPase defect. Indeed, since 75 to 80% of the dUTP synthesized in *E. coli* results from the deamination of dCTP (Karlström & Larsson, 1967), partial suppression of the *dut* mutation by a

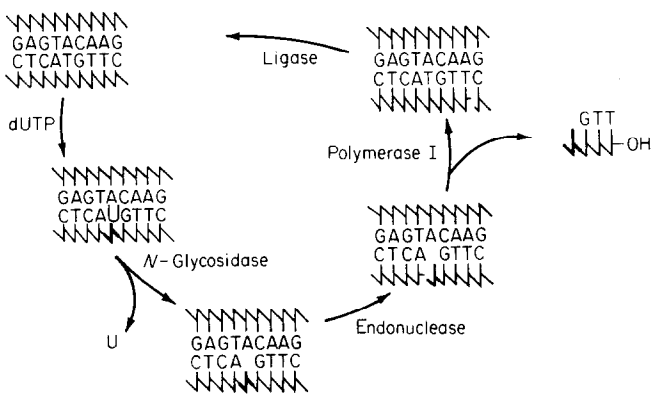

FIG. 9. Diagrammatic representation of the probable steps in the post-replication excision repair of uracil incorporated into DNA in dUTPase defective strains.

defect in the *dcd* locus alone might be anticipated. Deletion of the dCTP deaminase gene permits the *dut*-11 *pol*A12 strain to grow at non-permissive temperatures and suppresses the hyper rec phenotype of the *dut*-11 strain.

Although the excision repair process requires the concerted action of at least four different enzymes, it proceeds with great efficiency. Thus, when the *dut*-11 strain is pulsed with [^{3}H]thymidine for five to ten seconds, labeled 4 to 5 S fragments appear: however, at 30 seconds, most of the label is associated with large molecular weight DNA (>30 S).

dUTPase-defective mutants accumulate DNA fragments that closely resemble Okazaki fragments in that they appear transiently and can be readily incorporated into high molecular weight DNA. Their joining is also retarded in *pol*A$^-$ and *lig*$^-$ mutants. As judged by pulse-chase experiments, the fragments behave as though they are precursors to Okazaki fragments. It is therefore clear that simple pulse-chase experiments of this kind are not sufficient to define DNA replicative intermediates. Were uracil to be incorporated into DNA at the level of one or two molecules per 2000 nucleotides, a significant portion of the Okazaki fragments that appear in a pulse experiment could result from excision of the uracil and subsequent repair of the DNA.

Mutants of *E. coli* that are defective in DNA adenine methylase (*dam*) are also hyper rec (Marinus & Morris, 1974; Konrad, 1977). Thus, it is possible that other post-replication excision repair processes, in addition to that following uracil incorporation, might contribute to the pool of Okazaki fragments as observed in [^{3}H]thymidine pulse experiments.

The finding that the *ung*$^-$*dut*$^-$ double mutant is viable and lacks a discernible phenotype is surprising. One might have expected that a significant number of uracil residues incorporated in DNA as a result of a dUTPase defect would have an adverse affect on the replication and transcription of DNA. On the other hand, excision repair of uracil in the *ung*$^-$*dut*$^-$ double mutant may occur relatively slowly, so that repair would not be detected in the short pulses (ten seconds) used in our experiments. In particular, the *ung*$^-$ mutation might be leaky, or endonuclease V, which preferentially attacks uracil-containing DNA (Gates & Linn, 1977) could substitute for uracil *N*-glycosidase, but at a reduced rate. Finally, it is even possible that incorporation, excision and repair of uracil in DNA may serve a specific function in DNA replication.

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