

Transient accumulation of Okazaki fragments as a result of uracil incorporation into nascent DNA

(deoxyuridinetriphosphatase/*dnaS* gene/*sof* gene)

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ABSTRACT Strains of *Escherichia coli* with a mutation in the *sof* (*dnaS*) locus show a higher than normal frequency of recombination (are hyper rec) and incorporate label into short (4-5S) DNA fragments following brief [³H]thymidine pulses [Konrad and Lehman, *Proc. Natl. Acad. Sci. USA* 72, 2150 (1975)]. These mutant strains have now been found to be defective in deoxyuridinetriphosphate diphosphohydrolase (dUTPase; deoxyuridinetriphosphatase, EC 3.6.1.23), the enzyme that catalyzes the hydrolysis of dUTP to dUMP and PP_i. Reversion of one *sof*⁻ mutation to *sof*⁺ restores dUTPase activity and abolishes the accumulation of labeled 4-5S DNA fragments. Mutants initially isolated as defective in dUTPase (*dut*⁻) are also hyper rec and show transient accumulation of short DNA fragments. Both the *sof* and *dut* mutations are located at 81 min on the *E. coli* map, closely linked to the *pyrE* locus. The *sof* and *dut* loci thus appear to be identical.

A decrease in dUTPase as a consequence of a *sof* or *dut* mutation may result in the increased incorporation of uracil into DNA. Rapid removal of the uracil by an excision-repair process could then lead to the transient accumulation of short DNA fragments. It is possible that at least a portion of the Okazaki fragments seen in wild-type cells may originate in this way.

Although dUTP is the normal precursor of dTTP and can be incorporated efficiently into DNA by DNA polymerases (1), uracil is not normally found in DNA. At least two mechanisms prevent the permanent inclusion of uracil into DNA in *Escherichia coli*. First, an enzyme, deoxyuridinetriphosphate diphosphohydrolase (dUTPase; deoxyuridinetriphosphatase, EC 3.6.1.23), hydrolyzes dUTP to dUMP and PP_i (2, 3), thereby generating dUMP, the precursor in the *de novo* synthesis of dTTP, and destroying dUTP as a substrate for DNA replication (Fig. 1). Second, an excision-repair system detects and removes uracil residues that may have escaped the action of dUTPase and were misincorporated into DNA. Lindahl (4) has described an *N*-glycosidase that catalyzes the cleavage of the uracil-deoxyribose linkage in DNA, and nucleases, acting at the apyrimidinic acid site, might excise that region of the backbone (5, 6); the gap could be filled in by DNA polymerase I and DNA ligase to complete the repair process (7). Gates and Linn[§] have very recently identified an endonuclease that may also serve in removal of uracil residues by its specific capacity to hydrolyze uracil-containing DNA.

A defect in dUTPase would be expected to produce an increase in the intracellular pool of dUTP, and in addition, to block the predominant pathway of thymidine nucleotide biosynthesis, both of which should lead to an increased level of uracil in DNA. However, a group of dUTPase mutants recently isolated by Hochhauser and Weiss (8) contained no measurable

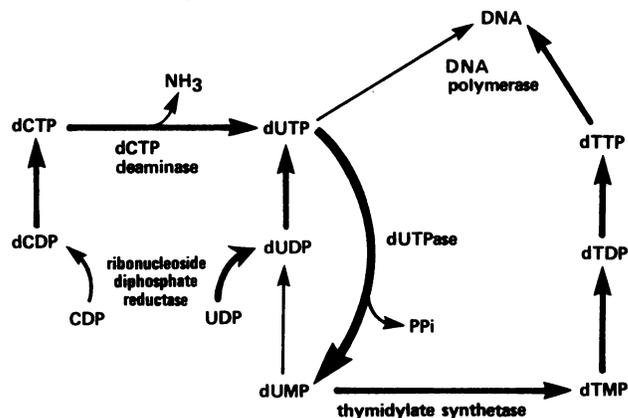


FIG. 1. Key role of dUTPase in *de novo* synthesis of dTTP and in elimination of dUTP.

uracil in their DNA, suggesting that the excision-repair system functions with extreme efficiency or that uracil was never incorporated.

The gene (or genes) for dUTPase (*dut*) has been located very near or at the *dnaS* locus (8). Inasmuch as mutants of *dnaS* accumulate short (4-5S) DNA fragments during brief pulses with [³H]thymidine (9), if *dnaS* mutants were deficient in dUTPase, they might incorporate uracil into their DNA. An efficient excision-repair system for removing the uracil would introduce nicks and gaps into newly synthesized DNA, thereby generating short DNA fragments; these could subsequently be covalently linked to the daughter strands.

In this paper we show that *dnaS* mutants, which we now refer to as *sof*, are deficient in dUTPase, and that *dut* mutants accumulate short DNA fragments. These traits are not only co-mutable, but they are also co-transducible and co-revertible, suggesting that the *dut* and *sof* genes are the same. It therefore appears that the DNA fragments seen transiently in cells harboring *sof* or *dut* mutations may indeed be a consequence of uracil incorporation into DNA. Furthermore, it is possible that some fraction of the Okazaki fragments observed in wild-type cells may have a similar origin.

MATERIALS AND METHODS

Bacterial Strains. Bacterial strains were all derived from *E. coli* K-12. Genetic nomenclature is that suggested by Bachmann *et al.* (10), except for *dut*, a gene symbol denoting mutations affecting dUTPase. The *sof*⁻ (*sof*-1, -2, and -3) strains are *pyrE*⁺ transductants of KS468 (F⁻ *metB*⁻ *thi*⁻ *pyrE*⁻ *lac*ΔMS286 φ80dII *lac*BK1 *str*^r) (9), a *lac* diploid strain used for testing for high frequency of recombination (hyper rec phenotype). Strain KS391 (Hfr Hayes *lac* MS286 φ80dII

Abbreviation: dUTPase, deoxyuridine triphosphate diphosphohydrolase.

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§ Gates, F. & Linn, S., *J. Biol. Chem.*, in press.

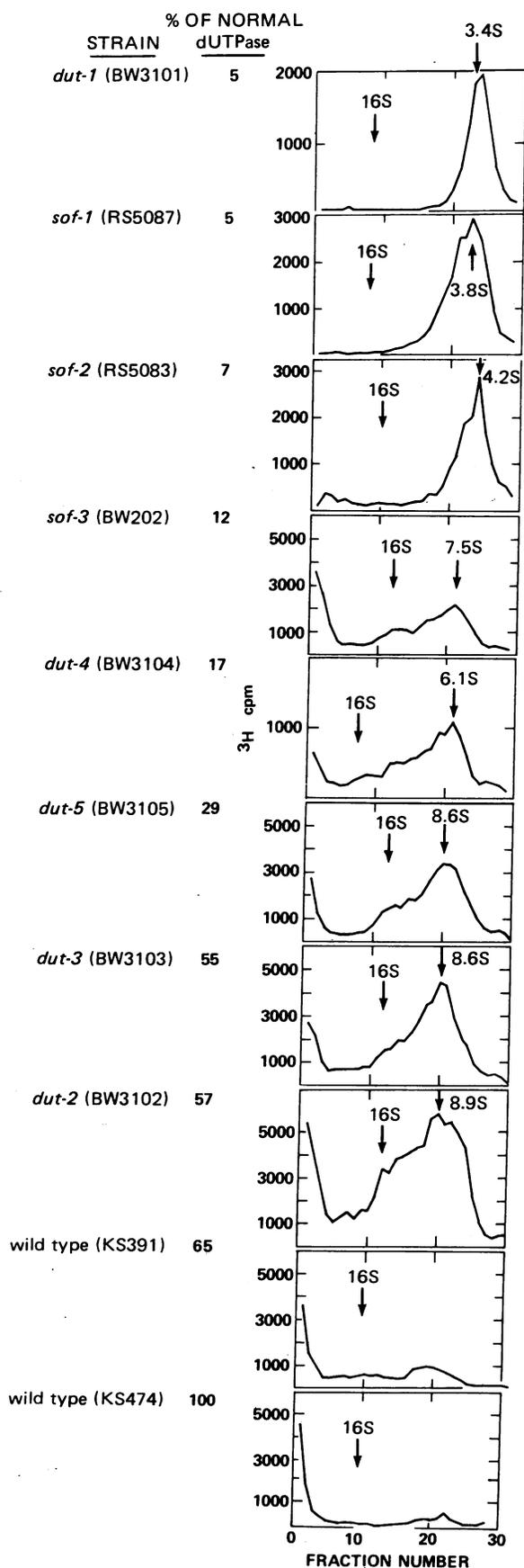


FIG. 2. *sof* and *dut* mutants accumulate short nascent DNA fragments. Values of dUTPase are relative to that of extracts of the

lacBK1 thi⁻ is the parent strain from which the *sof* mutants were initially derived (9). The *dut⁻* strains BW3001 to BW3005 were each obtained independently by treatment of strain AB1157 with nitrosoguanidine followed by a mass random testing of mutagenized clones for dUTPase activity.[†] Strains BW3101 to BW3105 are *pyrE⁺ dut⁻* transductants to KS468. AT2538 (*pyrE60*) was obtained from the *E. coli* Genetic Stock Center at the Yale University School of Medicine. Construction of the conditionally lethal double mutant BKT108 (*lacY⁻ str^r thyA⁻ rha⁻ polA12 sof-1*) will be described elsewhere[‡].

dUTPase Assays. dUTPase is highly specific for dUTP (2). Crude extracts of a wild-type strain (AB1157) hydrolyzed dUTP at a 20- to 25-fold greater rate than UTP or dTTP[†]. Moreover, the rate of hydrolysis of UTP or dTTP remained unchanged in a mutant, BW3001 (*dut-1*), which had 5% of wild-type dUTPase activity. Thus, the enzyme assayed in the crude extracts is dUTPase rather than a nonspecific nucleoside triphosphatase.

dUTPase deficiency was scored in large numbers of mutagenized clones and in transductants by semiquantitative microassay procedures (11). The assay is based on the release of ³²PP_i from [γ-³²P]dUTP, measured as ³²P not adsorbable to Norit (charcoal).[†] Quantitative assay for dUTPase were carried out by the following procedure. Ten microliters of extract (12) were added to 10 μl of a solution containing 0.4 M potassium phosphate (pH 6.5), 1.8 mM [³H]dUTP (Amersham/Searle), 20 mM MgCl₂, and 20 mM dithiothreitol. After incubation at 37° for 10 min, the reaction was stopped by the addition of 2 μl of 88% formic acid. An aliquot was applied to a strip of polyethyleneimine cellulose (Polygram CEL 300 PEI) together with unlabeled dUMP, dUDP, and dUTP, and the chromatogram was developed with 1 M formic acid containing 0.5 M LiCl at room temperature. The spots containing deoxyuridine nucleotide were cut out and their radioactivity was determined by liquid scintillation counting without prior elution. dUTPase activity was estimated as the fraction of the total ³H converted to dUMP.

Other Methods. The hyper rec phenotype was scored in derivatives of strain KS468 as described by E. B. Konrad (personal communication) by observing the relative number of *lac⁺* recombinants arising within a colony of the *lac* diploid mutant. Transductions with bacteriophage P1*vir*, pulse labeling of cells with [³H]thymidine, and alkaline sucrose density gradient centrifugation of DNA were performed as described previously (9). Protein was determined by the method of Lowry *et al.* (13).

RESULTS

Two groups of mutants were used; one, designated *dut⁻*, was isolated as defective in dUTPase activity and identified as such by assay of randomly chosen mutagenized clones (8); the other, designated *sof⁻*, was isolated initially because of an abnormally high recombination proficiency (hyper rec) and production of short (4-5S) DNA fragments (9).

The *dut* and *sof* Genes Are at the Same Locus; *sof* and *dut* Mutants Are Hyper Rec. The hyper rec character of *sof* mu-

[†] S. Hochhauser and B. W. Weiss, unpublished data.

[‡] B. K. Tye and I. R. Lehman, unpublished data.

wild-type strain KS474 (0.36 μmol of dUTP formed per min/mg of protein at 37°), which is taken as 100. Pulse labeling of cells with [³H]thymidine was carried out for 10 sec at 30° except for *dut-2* (BW3102), for which the temperature was 43°. Alkaline sucrose density gradient centrifugation was at 4° for 14 hr at 40,000 rpm in a Beckman SW41 rotor.

Table 1. Co-transduction of *dut* and *sof* with *pyrE*

Cross	Donor allele	Trait scored	Co-transduction frequency (%)
1	<i>dut-1</i>	dUTPase, hyper rec	89
2	<i>dut-2</i>	dUTPase	82
3	<i>dut-3</i>	dUTPase	85
4	<i>dut-4</i>	dUTPase	86
5	<i>dut-5</i>	dUTPase	90
6	<i>sof-1</i>	dUTPase, hyper rec	88
7	<i>sof-2</i>	hyper rec	98
8	<i>sof-3</i>	dUTPase	81

Phage P1 lysates of *dut*⁻ or *sof*⁻ mutants were used to transduce *pyrE*⁻ strains to uracil independence. From 74 to 180 *pyrE*⁺ transductants were repurified from each cross and tested for co-inheritance of dUTPase deficiency or of the hyper rec phenotype. Donors in crosses 1, 6, and 8 were *pyrE*⁺ *sof*⁻ or *pyrE*⁺ *dut*⁻ transductants of strain KS468. Donors in other crosses were the original mutants. Recipients were AT2538 for crosses 2 to 5 and KS468 for the other crosses.

tants was highly co-transducible with the *pyrE* locus (ref. 9; Table 1). The dUTPase deficiency of *dut* mutants was similarly co-transducible with *pyrE*. In Table 1, crosses 1 and 6, *pyrE*⁺ recombinants were scored both for dUTPase deficiency and for hyper rec phenotype; each dUTPase-deficient transductant was hyper rec, and vice versa. Although the hyper rec character was often difficult to score unambiguously, it was clearest in *sof-1* and *dut-1* strains, the mutants with the greatest dUTPase deficiency.

***dut* Mutants Accumulate Small DNA Fragments.** When pulsed at 30° for 5–10 sec with [³H]thymidine, the *sof-1* and *sof-2* mutants accumulate short DNA fragments with an average sedimentation coefficient of 4–5 S in alkaline sucrose density gradients (ref. 9; Fig. 2). The behavior of the *dut-1* mutant in such a pulse experiment was indistinguishable from the *sof-1* and *sof-2* mutants (Fig. 2).

In *sof* and *dut* mutants in which dUTPase activity was reduced 2- to 8-fold (*sof-3*, *dut-2*, -3, -4, and -5), pulse-labeled fragments also appeared. However, their average sedimentation coefficient was 8–10 S rather than 4–5 S. In addition, a substantial fraction of the label sedimented at >30 S (Fig. 2). The size of the labeled DNA in [³H]thymidine pulse experiments therefore appears to be related to the level of dUTPase.

***sof* Mutants Are Defective in dUTPase.** As shown in Fig. 2, extracts of all three *sof* mutants have significantly reduced dUTPase activity. In the case of *sof-1* and *sof-2*, the level (5–7%) was very close to that found in the *dut-1* strain, the most defective of the five *dut* mutants. The dUTPase activity of *sof* and *dut* mutants measured *in vitro* may not reflect accurately the level *in vivo*. dUTPase assays were carried out at dUTP concentrations that greatly exceeded the *K_m*, and, hence, changes in enzyme activity resulting from an alteration in *K_m* would not have been detected in these assays.

Restoration of dUTPase Activity Is Accompanied by Reversion of the Sof Phenotype in *polA12 sof-1*. Strains bearing either the *sof-1* or the temperature-sensitive *polA12* (14) mutation can grow at 30° and 43°. However, a strain (BKT108) that contains both the *sof-1* and *polA12* mutations, though viable at 30°, is unable to grow at 43°¹¹. Selection for temperature-resistant revertants of the double mutant yielded 30 such strains, all of which remained sensitive to methylmethane sulfonate at 43° and hence retained the *polA12* mutation (14). Extracts of six of the presumptive *sof*⁺ revertants were assayed for dUTPase activity. Of these, one (BKT108R9) showed nor-

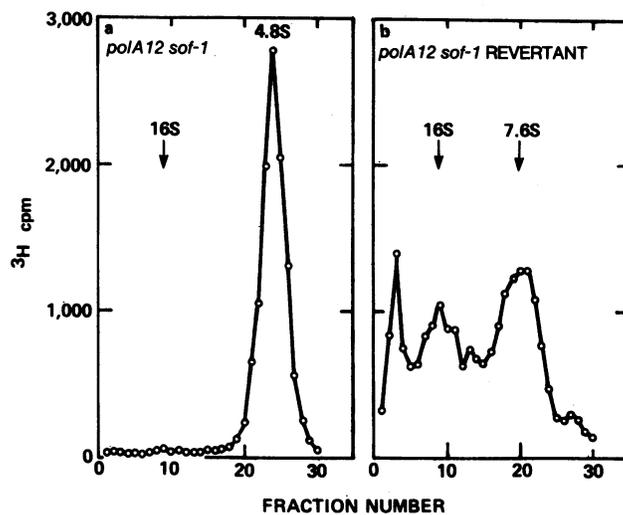


FIG. 3. Reversion of Sof phenotype in *polA12 sof*⁺ revertant of *polA12 sof-1* double mutant. Alkaline sucrose density gradients of DNA labeled with [³H]thymidine during a 10 sec pulse at 43° were centrifuged at 4° for 14 hr at 40,000 rpm.

mal activity (94% of wild type as compared with 5% in the double mutant). When this strain was pulsed with [³H]thymidine for 10 sec at 43°, the label had a sedimentation coefficient of 8–10 S, with some material at 30 S, as would be expected of a *polA* mutant (ref. 15; Fig. 3). Thus, in this instance, loss of the Sof phenotype was accompanied by restoration of dUTPase activity. Two of the remaining five strains also accumulated 8–10S fragments; however, their dUTPase activity was not significantly increased.

DISCUSSION

Although *sof* and *dut* mutants were isolated independently and by different screening procedures, it is clear that they represent mutations in the same genetic locus. Thus, *sof* and *dut* mutations (*t*) are closely linked to *pyrE* at 81 min on the *E. coli* map, (*tt*) are hyper rec, (*ttt*) are defective in dUTPase, and (*ttv*) accumulate short DNA fragments after brief pulses with [³H]thymidine. The size of the labeled fragments and the extent to which the fragments accumulate correlate well with the dUTPase activity. Furthermore, a revertant of one of the *sof* mutants has a normal dUTPase activity and no longer accumulates abnormally short DNA fragments.

It is unlikely that *sof* and *dut* represent two different genes within the same operon. Were this to be so, then all Sof and Dut mutations isolated thus far should be polar and lie within the gene proximal to the promoter, despite the fact that two completely different selection procedures were used for their isolation.

A review of the function of dUTPase (Fig. 1) provides a plausible explanation for the transient accumulation of small DNA fragments in mutants with reduced dUTPase activity. Under these conditions, available dUTP should be increased markedly relative to dTTP, and the frequency of uracil incorporation into DNA during replication should be correspondingly increased. The subsequent action of the *N*-glycosidase (4) and appropriate endonucleases (5, 6), which recognize and excise the uracil from DNA and in so doing produce a nick or gap, would result in the transient appearance of small DNA fragments in newly replicated DNA.^{**} The excision of uracil

^{**} Heat denaturation of the pulse-labeled DNA from *sof-1* followed by centrifugation in sucrose density gradients at neutral pH also yielded 4–5S fragments.

and the repair of the nick or gap produced under these conditions must be rapid because pulses greater than 10 or 20 sec significantly reduce the amount of labeled DNA fragments that accumulate in both *sof* and *dut* mutants.

One inference that can be reasonably drawn from our findings is that DNA fragments that are labeled during short pulses with [³H]thymidine and can be subsequently chased into high-molecular-weight DNA need not be replication intermediates. Inasmuch as dUTP is a normal product of pyrimidine nucleotide metabolism, low levels of uracil may be incorporated into DNA despite the presence of dUTPase. The size of the fragments generated as a result of uracil incorporation would depend largely upon the frequency with which such incorporation occurs. Spontaneous deamination of cytosine in DNA, which is believed to occur under physiological conditions, is yet another means for the generation of uracil residues in DNA (4, 16). Thus, it is possible that a significant proportion of the Okazaki fragments that are seen even in wild-type cells may result from excision-repair processes such as are described here.

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