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Role of Exonuclease III in the Base Excision Repair of Uracil-Containing DNA

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Mutants of *Escherichia coli* K-12 deficient in both exonuclease III (the product of the *xth* gene) and deoxyuridine triphosphatase (the *dut* gene product) are inviable at high temperatures and undergo filamentation when grown at such temperatures. In *dut* mutants, the dUTP pool is known to be greatly enhanced, resulting in an increased substitution of uracil for thymine in DNA during replication. The subsequent removal of uracil from the DNA by uracil-DNA glycosylase produces apyrimidinic sites, at which exonuclease III is known to have an endonucleolytic activity. The lethality of *dut xth* mutants, therefore, indicates that exonuclease III is important for this base-excision pathway and suggests that unrepaired apyrimidinic sites are lethal. Two confirmatory findings were as follows. (i) *dut xth* mutants were viable if they also had a mutation in the uracil-DNA glycosylase (*ung*) gene; such mutants should not remove uracil from DNA and should not, therefore, generate apyrimidinic sites. (ii) In the majority of the temperature-resistant revertants isolated, viability had been restored by a mutation in the dCTP deaminase (*dcd*) gene; such mutations should decrease dUTP production and hence uracil misincorporation. The results indicate that, in *dut* mutants, exonuclease III is essential for the repair of uracil-containing DNA and of apyrimidinic sites.

Exonuclease III of *Escherichia coli* has multiple enzymatic activities (29). It was originally described as a 3′→5′ exonuclease with an associated DNA-3′-phosphatase activity. Subsequently, it was discovered to have an exonucleolytic RNase H activity and to act as an AP endonuclease, i.e., a DNase attacking apurinic or apyrimidinic sites in DNA. Despite its abundance in *E. coli* and its many activities, its intracellular function remained obscure. Many *xth* (exonuclease III) mutants were isolated (17, 32), and although all of those tested were found to be simultaneously defective in the four enzyme activities (31), they appeared to have no marked physiological abnormality directly attributable to the mutation except, in some cases, for an enhanced rate of chromosomal recombination (34). In this study, we show that exonuclease III has a necessary role in a pathway for the base-excision repair of DNA, a process that generates apyrimidinic sites.

Base-excision repair depends on DNA glycosylases (13), enzymes that recognize specific unusual or damaged bases in DNA and remove them by catalyzing the hydrolysis of the glycosyl bond between that base and a deoxyribose. The free base is released, leaving behind an apurinic or apyrimidinic site (a base-free deoxyribose) in the intact DNA backbone. The first such enzyme to be characterized was uracil-DNA glycosylase of *E. coli* (14), the product of the *ung* gene (5), which removes uracil residues from DNA. Such residues result commonly from the spontaneous hydrolytic deamination of DNA cytosine (15) and from the misincorporation of dUTP in place of dTTP, a consequence of the formation of large amounts of dUTP as an intermediate in the biosynthesis of thymidylate (19). Figure 1 shows a scheme for the base-excision repair of uracil-containing DNA and for the proposed role of exonuclease III. First, the glycosylase removes uracil, leaving an apyrimidinic site. Exonuclease III then acts as an AP endonuclease; it is known to cleave 5′ to a base-free deoxyribose (31), as indicated. DNA polymerase I can then remove the base-free sugar (9) via its 5′→3′ exonuclease activity (12) while it fills in the resulting gap via polymerization. DNA ligase (12) completes the repair. Although exonuclease III is only one of several AP endonucleases in *E. coli*, studies with mutants have shown that it is responsible for 80 to 85% of such activity in crude extracts (29).

Evidence for much of this pathway (Fig. 1)

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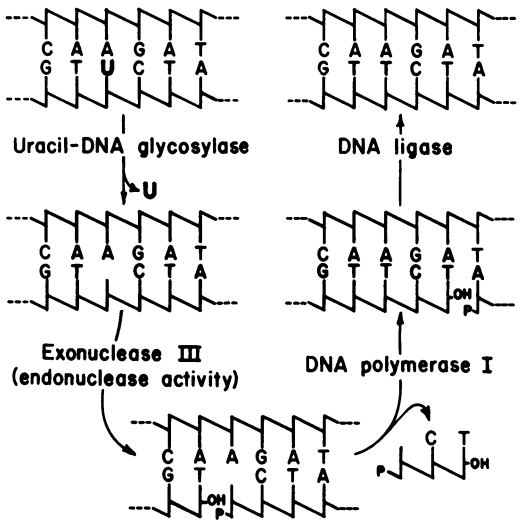

FIG. 1. Proposed role for the endonucleolytic activity of exonuclease III in the repair of uracil-containing DNA.

has been provided by studies on *dut* mutants (10), which are deficient in dUTPase (deoxyuridine triphosphatase; EC 3.6.1.23) and which therefore have a high rate of misincorporation of uracil into DNA in place of thymine. Thus, *dut* mutants display an excessive fragmentation of nascent DNA consistent with excision repair (24). *dut ung* double mutants accumulate uracil in their DNA (26), indicating an essential role for the glycosylase. *dut polA* and *dut lig* combinations are conditionally lethal under circumstances in which the single mutations are not (23), suggesting that DNA polymerase I and DNA ligase are part of the repair pathway for uracil-containing DNA. We shall now provide analogous evidence for the proposed role of exonuclease III, based on the conditional inviability of *dut xth* mutants. Some of our results appeared in a preliminary report (31).

MATERIALS AND METHODS

Bacterial strains. All strains analyzed in this study were derived from the *E. coli* K-12 strain Hfr KL16. They were constructed by phage P1 transduction from the strains listed in Table 1. *xth* mutations were introduced first, followed by *ung* and then *dut*. Congenic Δ*xth* and *xth*$^+$ strains were constructed from BW9101 and KL16, respectively. Strain BW280 was the donor of *ung-1*. Selection was for tetracycline resistance (i.e., transfer of the nearby Tn*10*); *ung* was scored by sensitivity or resistance to uracil-containing T5 phages (27) and confirmed by enzymatic assay (14). *ung nad* Tn*10* recombinants were picked, and the nearby *nad* and Tn*10* markers were then replaced by wild-type alleles via transduction with selection for nicotinate independence (*nad*$^+$). *dut* markers were transferred from strain BW231 or BW288 by selecting for the transduction of the nearby Tn*10* at 25°C. The *dut* mutations were scored by uracil sensitivity and confirmed by enzymatic assay (10). For the construction of strains bearing *xth* alleles other than Δ*xth*, AFT20 was used as the transductional recipient, and its deleted *xthA-pncA* segment was replaced with those from other *xth* mutants by selecting for nicotinamide utilization (*pnc*$^+$). The *nadB* and Tn*10* markers were then crossed out as described above. Congenic strains bearing wild-type alleles were picked from the same transductional crosses in which their mutant counterparts were generated.

Media and transductions. The following were as previously described: nutrient and minimal media and supplements (10, 32), transductions with bacteriophage P1 *vir* (10, 32), selections for tetracycline resistance using 25 μg of the drug per ml (10), and selection for and scoring of *nad* and *pnc* markers (32). Unless otherwise stated, cultures were grown in nutrient broth supplemented with 0.2% glucose.

Isolation of revertants. A *dut-1* Δ*xth* strain, constructed as described above, was plated out for individual colonies on nutrient agar at 25°C. Then, 192 clones were picked and grown at 25°C in individual wells of microwell plates (30). Droplets of each culture were transferred via a 48-prong inoculator (30) to the surface of nutrient agar plates that were then incubated at 37°C. Colonies that appeared were restreaked to single clones at 37°C. One revertant was picked from each of the 92 microwell cultures that produced them.

Enzyme assays. *xth* was scored with the exonuclease assay for exonuclease III, whereas quantitative estimates of residual activity were based on the more specific DNA-3′-phosphatase assay, using a terminally labeled substrate (18). The following were as described: dUTPase assays (assay D, reference 10), uracil-DNA glycosylase assay (14), preparation of extracts and assays for dCTP deaminase (24), and protein determinations (2). Enzyme activities are expressed as the ratio of the specific activity (units per

TABLE 1. Bacterial strains

Strain	Relevant genotype	Source/reference[a]
Hfr KL16	Wild type; prototroph	32
BW9101	Δ(*xthA-pncA*) DE90	Spontaneous deletion mutant of KL16 (32)
BW280	*ung-1 nadB7 zfe-208*::Tn*10*	By transduction from a strain of B. K. Duncan into Hfr KL16
BW231	*dut-1 zib-205*::Tn*10*	10
BW288	*dut-11 zib-205*::Tn*10*	Constructed like BW231
AFT20	Δ(*xthA-pncA*) DE90 *nadB7 zfe-208*::Tn*10*	Transduction: BW280 × BW9101 → Tet^r
BW9081	*xthA1*	32
BW9093	*xthA3*	32
BW2031	*xthA11*	23
BW2021	Revertant of *xthA11*	23

[a] In some cases, references are to the mutant alleles rather than to the specific strains.

milligram of protein) in the mutant extract to that of the wild-type control.

Measurements of cell length. Cultures were grown at 25°C to saturation, diluted 100-fold into prewarmed media at 37°C, and incubated with shaking for 3.5 h. Samples were spread on glass slides, heat fixed, and stained with methylene blue. Cell lengths were measured, with a Numonics electronic digitizer, from projected photomicrographs that were calibrated with a stage micrometer.

RESULTS

Inviability of *dut xth* mutants. P1-mediated transduction was used to construct a set of congenic strains containing *dut*, *xth*, and *ung* alleles in various combinations. Their genotypes were confirmed by enzyme assays. The strains were propagated at 25°C, and their viability was measured at higher temperatures (Table 2). Strains that bore a single mutation had no apparent growth defect, except for the *dut-1* strain, which had a slightly reduced survival at 42°C. Both the Δ*xth dut-1* and the Δ*xth dut-11* strains, however, were almost completely inviable at 42°C.

The Δ*xth dut-1* strain was more temperature sensitive than its Δ*xth dut-11* counterpart, and this result was consistent with the known relative leakiness of *dut-11*. At 37°C, the *dut-1* strains had <1% of wild-type dUTPase activity, whereas the *dut-11* strains had 8 to 11%. Previously, a *dut-1* strain was also found to be more defective than a *dut-11* mutant in vivo during studies on the fragmentation of nascent DNA (23, 24), although, contrary to our present findings, they appeared to have the same level of enzyme activity. The residual activity of the *dut-1* dUTPase (5% at 25°C) was also previously shown to be temperature sensitive (10). The conditional lethality of a Δ*xth dut* strain thus appears to be related to its residual dUTPase level.

TABLE 2. Temperature sensitivity of *dut* Δ*xth* and suppression by *ung*

Genotype[a]	Relative efficiency of plating[b]		
	30/25°C	37/25°C	42/25°C
Wild-type	1.2	1.2	1.2
Δ*xth*	0.9	1.1	1.2
dut-1	0.9	0.9	0.4
Δ*xth dut-1*	0.2	2×10^{-5}	2×10^{-5}
Δ*xth dut-11*	0.6	0.1	2×10^{-4}
Δ*xth dut-1 ung-1*	1.0	0.7	0.5

[a] Wild type = Hfr KL16 *zib-205*::Tn*10*. Derivatives have additional markers as indicated. Δ*xth* = Δ(*xthA-pncA*) DE90.

[b] Saturated cultures grown at 25°C were diluted and spread on nutrient agar media at the indicated temperatures.

TABLE 3. Conditional lethality of various *xth* mutations in a *dut-1* strain

xth allele	Relative efficiency of plating[a]		
	30/25°C	37/25°C	42/25°C
xthA1	1.1	0.9	1×10^{-5}
xthA3 (Ts)[b]	1.1	0.8	4×10^{-6}
xthA9	0.2	2×10^{-5}	1×10^{-5}
xthA11	0.1	2×10^{-5}	1×10^{-5}
xthA11rev[c]	1.2	0.9	0.5

[a] The relative plating efficiency of all corresponding *dut*$^+$ derivatives (not shown) ranged from 0.8 to 1.2 at all temperatures.

[b] The *xthA3* allele specifies a temperature-sensitive protein (18).

[c] This allele is a partial revertant of *xthA11*. It was obtained from BW2021, a strain that had regained 77% of wild-type exonuclease III activity (33).

Viability of a Δ*xth dut ung* strain. *dut* mutants frequently incorporate uracil into DNA in place of thymine. This uracil can be removed by uracil-DNA glycosylase (the product of the *ung* gene), leaving apyrimidinic sites (Fig. 1) which, if unrepaired, might be lethal. The lethality of the Δ*xth dut* combination can therefore be explained by a probable need for exonuclease III in the repair of apyrimidinic sites. If this hypothesis is correct, then an *ung* mutation, by blocking the formation of these sites, should restore the viability of an *xth dut* mutant; *dut ung* mutants are known to retain uracil in their DNA while remaining viable (26). Accordingly, a Δ*xth dut ung* mutant was constructed and its genotype was confirmed by enzymatic assays. The addition of the *ung* mutation almost completely reversed the lethality of the Δ*xth dut* combination (Table 2). The results strongly suggest that exonuclease III is needed for the efficient repair of apyrimidinic sites arising from uracil-containing DNA.

Other *xth* mutants. The *xth* deletion mutant used in the above experiments lacks the *xthA-pncA* region, which covers a recombinational distance of 0.5 min on the genetic map of *E. coli* (1, 32). We considered the possibility that an unknown gene in this interval was responsible for the effects we attributed to *xth*. We therefore examined other *xth* alleles, ones that had been obtained with nitrosoguanidine mutagenesis and which were therefore probably point mutations. One of the alleles (*xthA3*) specifies a temperature-sensitive exonuclease III (8) and must therefore be mutated within the structural gene for that enzyme. Again, the *dut xth* strains were temperature sensitive (Table 3). As an additional control, an *xth* revertant allele was used; it did not confer temperature sensitivity on the *dut* mutant.

***dut-1* mutants are thymidine auxotrophs.** To

understand the reversion experiments described below, we must first consider the role of dUTPase in thymidylate metabolism. In *E. coli*, dUTP is believed to be a precursor for most if not all of the thymidylate that is synthesized de novo (19). dUTP is degraded by dUTPase to PP_i and dUMP, which is the substrate for thymidylate synthetase. Although the original *dut* strains were not thymidine auxotrophs (10), we found that, if the *dut-1* allele is transduced into other strains that are then propagated at low temperature in the presence of thymidine (100 μg/ml), the recombinants have a temperature-sensitive requirement for thymidine. When propagated at 37°C in the absence of thymidine, they tended to revert to prototrophy while remaining dUTPase deficient, thus explaining our previous failure to observe this defect. A *dut-1* strain, for example, was a prototroph at 30°C but would not grow in minimal medium at 37 or 42°C unless thymidine were added. Thymine could be substituted for an equimolar concentration of thymidine, but the resulting colonies at 42°C were smaller, probably because thymine is utilized less efficiently than thymidine by wild-type (*thyA*$^+$) *E. coli* (19). Our results are consistent with those of Warner et al. (26), who reported that the filamentation of *dut-1* cells at high temperature could be reduced by the addition of thymidine.

Temperature-resistant revertants of an *xth dut* strain. Revertants of a Δ*xth dut-1* strain were selected by their ability to grow on nutrient media at 37°C. From 192 parental clones, 92 independent revertants were isolated and then characterized with respect to their growth requirements and enzyme levels. They fell into three general classes (Table 4). The first were Dut$^+$ revertants; they had regained most of their dUTPase activity and lost their requirement for thymidine. This result further confirms the hypothesis that the temperature sensitivity of the parental strain was due to *dut-1* and not to a hidden mutation in a closely linked gene.

The second, and major, revertant class comprised thymidine auxotrophs. Five of the six strains tested were *dcd* mutants; i.e., they had acquired a deficiency of dCTP deaminase. One of these also required histidine and was probably, therefore, a *his-dcd* deletion mutant. Such mutants have been found previously among temperature-resistant *dut polA* revertants (23). The *dcd xth dut* triple mutants had a greater defect in thymidylate metabolism than the parent *xth dut* strain; their thymidine requirement could be satisfied by 0.4 mM thymine at 30°C but not at 42°C. These results were consistent with those of previous studies that concluded that dCTP deaminase produces 75% of cellular dUTP and that it is on the major pathway for the synthesis de novo of thymidylate (19). They are also consistent with our hypothesis that the lethality of *dut* in an *xth* mutant stems from an enhanced misincorporation of dUTP into DNA. Thus, a mutation that reduced dUTP formation reduced lethality.

TABLE 4. Temperature-resistant revertants of a Δ*xth dut-1* strain

Class	No.	Enzymatic analysis	
		No. tested	Result
I. Prototrophs	7	3	All *dut*$^+$ (65–75% of wild-type activity)
II. Thymidine auxotrophs	77	6	5 *dcd* (≤10% of wild-type activity) 1 still *xth dut dcd*$^+$ *ung*$^+$
III. Unidentified auxotrophs	8	0	

Among the revertants that were still thymidine auxotrophs, there was one that had parental enzyme levels; i.e., it still appeared to be *xth dut dcd*$^+$ *ung*$^+$. It might have been, for example, a pseudorevertant with an unstable dUTPase. It was not further characterized. In addition, there were eight slowly growing revertants that had an unidentified auxotrophic defect and that will be the objects of further study. Unexpectedly, none of the phenotypic revertants examined were Ung$^-$.

Filamentation of *xth dut* mutants. In *E. coli*, cell division is coupled to chromosomal replication; bacteria that undergo chromosomal damage or that do not complete a round of DNA replication fail to divide (as reviewed in reference 11). As they continue to grow without division, they elongate and become filamentous. Because filamentation can be a sensitive indication of DNA damage, we measured the lengths of our mutant cells (Fig. 2). In agreement with the results of Warner et al. (26), cells possessing the *dut-1* mutation alone were two to three times the length of *dut*$^+$ cells during logarithmic growth. When grown to saturation, however, they were the same length as wild-type cells (data not shown), presumably because as they near saturation, DNA repair and cell division can keep pace with their slower growth. Whereas the *dut-1* cells showed minor elongation and the *xth* mutant showed none, the Δ*xth dut-1* double mutants were 5 to 20 times the length of wild-type cells when grown at 37°C. Their filamentation, however, was abolished by the addition of an *ung* mutation. These results were consistent with the survival data (Table 2).

DISCUSSION

Mutant strains of *E. coli* deficient in both exonuclease III and dUTPase are inviable at

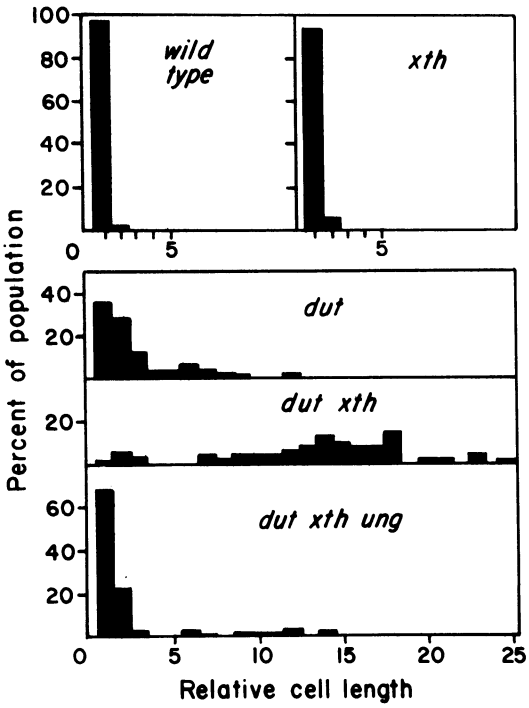

FIG. 2. Distribution of cell lengths in populations of mutants grown at 37°C. Relative lengths are the measured lengths divided by 1.9 μm, the mean length of wild-type (*dut*$^+$ *xth*$^+$ *ung*$^+$) cells. Over 50 cells were measured in each culture. *dut*, *xth*, and *ung* refer to the *dut-1*, Δ*xth*, and *ung-1* markers, respectively. The strains were those described in Table 2.

high temperatures: they fail to form colonies on agar and they undergo extreme filamentation in liquid culture. Their temperature sensitivity is presumed to be due to that of the residual dUTPase activity. This phenotype was demonstrated with cells bearing several different mutant *xth* alleles, including a deletion, and with two *dut* alleles. There was an apparent correlation between the degree of enzymatic deficiency and the degree of temperature sensitivity of each strain.

Two lines of evidence support the hypothesis that the conditional inviability of *dut xth* strains is due to events following the misincorporation of uracil, a consequence of elevated dUTP pools in *dut* strains (26), and not due to an unknown effect of the *dut* mutation. First, their viability was restored by an *ung* mutation, which reduces the excision of uracil from DNA and thereby reduces the subsequent formation of apyrimidinic sites. Second, their viability was restored by *dcd* mutations, which are known to reduce the dUTP pool. The restoration of viability by the *ung* mutation also indicates that is not the production of uracil-containing DNA that is in itself lethal in *xth* mutants, but rather is the resulting accumulation of apyrimidinic sites during the attempted repair.

There have been several studies that tested *xth* mutants for the lethal effects of various physical or chemical agents that damage DNA, such as alkylating agents (4, 17, 18, 33), nitrous acid (4), bisulfite (22), and ionizing radiation (18, 20). These agents are capable of promoting apurinic or apyrimidinic sites; such sites result from the spontaneous or enzymatic hydrolysis of the altered nucleoside residues in DNA. Specific glycosylases, for example, will remove alkylated adenine residues, bases that have been deaminated by nitrous acid or bisulfite, and bases that have been damaged by X-irradiation (3, 13). Studies measuring the sensitivity of *xth* mutants to these agents are difficult to interpret. The sensitivity of the mutants was variable and not related to their degree of enzymatic defect. Moreover, some of the conclusions reached by these studies were based on comparisons between heavily mutagenized strains and control strains that were either unmutagenized or otherwise noncongenic. Some of the properties attributed to *xth* mutants may have been due, therefore, to hidden secondary mutations. For example, strain BW2001, the original *xth-11* isolate, had a marked sensitivity to methyl methane sulfonate that coreverted with the enzymatic defect (33). Some *xth-11* transductants, however, were only slightly sensitive to methyl methane sulfonate (B. Weiss, unpublished data). Similarly, Seeberg and Steinum (20) concluded that the X-ray sensitivity of that strain was due to a secondary mutation. Perhaps the most valid data, therefore, were those obtained when cells bearing the deletion mutation Δ(*xthA-pncA*) were compared with congenic *xth*$^+$ strains. The deletion mutation is not only the tightest one, but also the only one studied that has been obtained without a mutagen. The Δ*xth* strains that were examined were no more sensitive to methyl methane sulfonate (4), nitrous acid (4), or X-irradiation (20) than their congenic *xth*$^+$ counterparts. They did appear, however, to be more sensitive to killing by sodium bisulfite (22). Results obtained with these chemical agents, however, are often difficult to interpret because they each produce multiple types of damage, some of which are obscure (6, 21), and their lethality is not always related to their ability to produce mutagenic alterations of DNA. In this study, we have chosen to use the *dut* mutation, rather than a chemical agent, to alter DNA in vivo. The advantage of this approach is that we expect to produce only a single, well-defined lesion in DNA, namely, the A:U base pair.

Warner et al. (26) determined that a 37°C an *ung-1 dut-1* mutant replaced at least 13% of its DNA thymine residues with uracil and remained

viable. An *ung*$^+$ *dut xth* mutant might therefore be expected to generate up to an equivalent amount of unrepaired apyrimidinic sites in its DNA. Such DNA would be an inefficient template for replication, thus explaining the conditional inviability of the strain. In the repair of the uracil-containing DNA, the most obvious role for exonuclease III is as an AP endonuclease; it is responsible for 80 to 85% of such activity in *E. coli*. Our results, however, do not preclude the possibility that the exonuclease activity may be required at a later stage in the excision repair. It has been suggested, for example, that its exonuclease activity might prevent premature sealing of the incised strands by DNA ligase (9) or that it might be needed to remove DNA–3′-deoxyribose termini generated by endonuclease III (25). The gap created by the exonuclease also might provide a better priming site for DNA polymerase I, or it might stimulate recombinational repair.

There are other endonucleases that might function in the repair of uracil-containing DNA in *E. coli*. Endonuclease IV is an AP endonuclease that is responsible for about 10% of such activity in *E. coli* (16). Endonuclease III (8) is believed to cleave 3′ to apurinic or apyrimidinic sites (25). Endonuclease V cleaves uracil-containing DNA (7). Our findings do not rule out significant exonuclease III-independent pathways of repair for uracil-containing DNA; they indicate only that when there is a high degree of uracil misincorporation, as in *dut* mutants, exonuclease III is required. The relative contribution of other endonucleases to these pathways can best be determined after the appropriate mutants are isolated.

Our discovery of the inviability of *dut xth* mutants (31) provided the first evidence for a biological role for exonuclease III and suggested that its AP endonuclease activity provides at least part of its reason for being. A model has been presented (28) in which the multiple activities of the enzyme are catalyzed by the same active site. It is possible, therefore, that the AP endonuclease activity might be the sole essential function of the protein. The other activities that we detect in vitro, namely, the DNA–3′-phosphatase and exonuclease (including the RNase H) activities, might merely be manifestations of the broad specificity of this phosphoesterase and of little consequence to the cell.

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