Identification of RNase T as a High-Copy Suppressor of the UV Sensitivity Associated With Single-Strand DNA Exonuclease Deficiency in *Escherichia coli*

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ABSTRACT

There are three known single-strand DNA-specific exonucleases in *Escherichia coli*: RecJ, exonuclease I (ExoI), and exonuclease VII (ExoVII). *E. coli* that are deficient in all three exonucleases are abnormally sensitive to UV irradiation, most likely because of their inability to repair lesions that block replication. We have performed an iterative screen to uncover genes capable of ameliorating the UV repair defect of *xonA* (ExoI$^-$) *xseA* (ExoVII$^-$) *recJ* triple mutants. In this screen, exonuclease-deficient cells were transformed with a high-copy *E. coli* genomic library and then irradiated; plasmids harvested from surviving cells were used to seed subsequent rounds of transformation and selection. After several rounds of selection, multiple plasmids containing the *rnt* gene, which encodes RNase T, were found. An *rnt* plasmid increased the UV resistance of a *xonA xseA recJ* mutant and *uvrA* and *uvrC* mutants; however, it did not alter the survival of *xseA recJ* or *recA* mutants. RNase T also has amino acid sequence similarity to other 3′ DNA exonucleases, including ExoI. These results suggest that RNase T may possess a 3′ DNase activity capable of substituting for ExoI in the recombinational repair of UV-induced lesions.

IN *Escherichia coli*, the excision of DNA lesions such as UV photodimers, or bulky DNA adducts such as benzo[a]pyrene, is mediated by the nucleotide excision repair (NER) system (Van Houten 1990; Sancar 1996). This system, composed of the UvrABC complex of proteins, specifically detects and removes DNA lesions from duplex DNA. However, lesions on single-strand DNA (ssDNA) that arise during the process of DNA replication are refractory to NER. Lesions on ssDNA may either be introduced after strand separation during DNA replication, or they may be derived from preexisting lesions left unrepaired by the NER system. The latter is more likely to occur if the NER system is saturated by a large number of lesions. Even small lesions, such as pyrimidine dimers, are impediments to DNA polymerase and stall replication. Replication will typically restart downstream of such lesions, leaving ssDNA gaps (Rupp 1996). Although error-prone lesion bypass by the polymerase is possible via SOS induction of the UmuCD′ complex of proteins, this system does not contribute substantially to UV survival, as *umuCD* mutants show only modest sensitivity to UV (Kato and Shinoura 1977). Lesions within the replication fork can be more faithfully repaired by a RecA-dependent recombination process termed "postreplicational repair" (Rupp *et al.* 1971; Rupp 1996). Postreplicational repair uses information from an intact sister chromosome to fill in the gap left in the wake of a stalled replication fork. Once the gap is filled, the resulting duplex DNA is subject to repair by the NER system. In addition to RecA, there are a number of other proteins in *E. coli*, including RecBCD (helicase/exonuclease), RecFOR (RecA filament loading and stabilization proteins), and RuvABC (Holliday junction processing), that are involved in the recombinational repair process (reviewed in Kowalczykowski *et al.* 1994). Mutation of any of these functions results in varying degrees of sensitivity to UV irradiation.

We previously discovered that *E. coli* deficient in the three known ssDNA-specific exonucleases (ssExos)—RecJ, exonuclease I (ExoI), and exonuclease VII (ExoVII)—are sensitive to UV irradiation (Viswanathan and Lovett 1998). RecJ exonuclease and ExoI, encoded by the *recJ* and *xonA* genes, respectively, have opposing directionalities of DNA degradation: RecJ degrades ssDNA with 5′–3′ polarity (Lovett and Kolodner 1989), whereas ExoI degrades with 3′–5′ polarity (Lehman and Nussbaum 1964). Exonuclease VII, a two-subunit enzyme encoded by the *xseA* and *xseB* genes, has dual polarity, degrading from either DNA end (Chase and Richardson 1974). We proposed that ssExos may be necessary to aid in the recombinational repair of gaps caused by UV-induced lesions within the replication fork. A modest recombination deficiency has been demonstrated for mutants in ssExos by various assays (Miesel and Roth 1996; Razavy *et al.* 1996; Viswanathan and Lovett 1998). RecA requires a minimum of 50 nucleotides of ssDNA on which to bind and nucleate the formation of a RecA filament for recombinational strand exchange (Brenner *et al.* 1987). In situations where there is a limited amount of ssDNA, as might be the case

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surrounding a lesion, RecA may not have sufficient room to bind. In such cases, ssExos, with the aid of DNA helicases, could widen the gaps and allow RecA to begin recombinational repair. Exonucleases may also aid recombination postsynaptically by degrading displaced DNA strands, thereby stabilizing heteroduplex recombination intermediates.

We have devised a high-copy suppressor screen to identify *E. coli* genes, especially other ssExos, capable of compensating for the UV repair defect of a RecJ$^-$ ExoI$^-$ ExoVII$^-$ strain. In general, ssExos are poorly expressed proteins (Chase *et al.* 1986; Phillips and Kushner 1987; Lovett and Kolodner 1991); therefore, a high-copy screen was chosen so that poorly expressed genes could be detected. Furthermore, an iterative selection scheme was used to enrich for clones providing gain of function, thereby limiting the number of clones to be analyzed. Using this screening method, we have uncovered a single gene, *rnt*, which is capable of increasing the UV survival of a RecJ$^-$ ExoI$^-$ ExoVII$^-$ mutant, but not that of a RecJ$^-$ ExoVII$^-$ mutant. RNase T, the product of the *rnt* gene, has been identified previously as an RNase involved in the exonucleolytic processing of tRNA (Deutscher *et al.* 1985) and 5S RNA (Li and Deutscher 1995). When transformed into NER-deficient strains, an *rnt*-containing plasmid increases UV resistance. However, in stark contrast, the same plasmid provides no measure of protection to strains deficient in various recombination functions. These results suggest that *rnt*'s effect on UV survival is caused by an enhancement of recombinational repair. From its ability to specifically compensate for an ExoI defect, we conclude that RNase T likely possess a DNase activity capable of degrading DNA with 3′–5′ polarity, similarly to its RNase activity.

MATERIALS AND METHODS

Bacterial strains, media, and antibiotics: Isogenic strains, listed in Table 1, are derived from BT199 and were constructed by P1 transduction (Miller 1992). Strains were grown at 37° in LB medium composed of 1% Bacto-tryptone, 0.5% yeast extract, 0.5% NaCl; plate media contained 1.5% agar. Plate minimal medium consisted of 56/2 salts (Willetts *et al.* 1969) supplemented with 0.2% glucose, 50 μg/ml of the appropriate required amino acids, and, for plates, 2% agar. Guanosine was added to Luria broth (LB) and minimal media at 25 μg/ml. The antibiotics ampicillin (Ap), tetracycline (Tc), and kanamycin (Km) were added at concentrations of 100, 15, and 60 μg/ml, respectively. LB calcium glucose (LCG) medium for the preparation of P1 lysates and transductions consisted of LB medium supplemented with 2 mm calcium chloride, 50 μg/ml thymine, 0.2% glucose, and 1.2% agar; LCG top agar contained 0.7% agar.

Genomic library construction: Genomic DNA was isolated from *recJ2052*::miniTn*10kan* Δ*xonA300::cat* Δ*xseA* strain, STL 2971 (Viswanathan and Lovett 1998), using the Qiagen genomic tip 500-G. The purified chromosomal DNA was subsequently digested with *Sau*3A (New England Biolabs Inc., Beverly, MA) to generate a range of fragments between 100 bp and 10 kb. The digested fragments were separated on a 30-ml 10–40% sucrose gradient and centrifuged in an SW28 rotor (Beckman, Fullerton, CA) at 89,500 *g* for 26 hr. The gradient was fractionated into 1-ml samples and analyzed by agarose gel electrophoresis to determine the size range of fragments in each sample. Fractions containing DNA from 4 to 10 kb in range were pooled and dialyzed against Tris-EDTA, pH 8.0. Plasmid pBSSK$^-$ was digested with *Bam*HI and treated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis) to reduce intramolecular religation. Two-hundred nanograms of *Bam*HI-digested and dephosphorylated pBSSK$^-$ was ligated to 80 ng of a size-selected insert in a 20-μl reaction with 200 units of T4 DNA ligase and incubated at 16° for 16 hr. The ligation reaction was transformed by electroporation (Dower *et al.* 1988) into XL1-Blue (F′::Tn*10 proAB lacIq lacZ*ΔM15/*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lac*; Stratagene, Inc., La Jolla, CA) cells. Transformants were selected on LB + Ap medium. The total number of initial transformants was 2.4×10^4, with 90% insertion as determined by blue/white color screen. The library was subsequently amplified in LB + Ap liquid medium, and plasmid DNA was isolated by the alkaline/sodium dodecyl sulfate method and purified by CsCl centrifugation (Sambrook *et al.* 1989). The average insert size after amplification was 5 kb.

Enrichment screen: A total of 1.5 μg of genomic library (or in subsequent rounds, enriched plasmid DNA) was transformed by electroporation into STL2701. Transformants were allowed to recover nonselectively for 1 hr and were then transferred to selective medium containing Ap. This culture was allowed to grow shaking for 5 hr at 37°. The cells were serially diluted in 56/2 salts and plated on LB + Ap medium to determine the colony-forming units per milliliter of the culture; meanwhile, the cultures were kept at 4°. The next day, 10^8 cells were plated on LB + Ap medium, such that each plate had 4.0×10^6 cells. Plates were immediately irradiated with a 20 J/m^2 dose of UV (254 nm) light and incubated at 37° in the dark overnight. The next day, surviving colonies on the irradiated plates were pooled by washing the plates with LB liquid medium. Plasmid DNA was isolated from the pooled cells using the Wizard midi prep kit (Promega, Madison, WI). This DNA was then used for the next round of transformation and irradiation. Fractional survival of the population at each iteration was determined from serial dilutions of irradiated and unirradiated cells taken from the transformation cultures at the time of plating. Individual isolates were selected from the sixth round of transformants and tested quantitatively for UV survival at 20 J/m^2. Clones with UV survival greater than the vector control were sequenced. Sequence determination of the endpoints of the plasmid inserts employed standard M13 forward and M13 reverse primers using the SequiTherm Excel sequencing kit (Epicentre Technologies), following the methods recommended by the manufacturer.

UV survival assays: Plasmids were transformed into various strains (Table 1) by chemical transformation (Chung *et al.* 1989) selecting on LB + Ap medium. Single colonies were grown in LB liquid medium to exponential stage (OD_{600} = 0.4–0.5), serially diluted in 56/2 buffer, and plated on LB + Ap plates. Plates were immediately irradiated with varying doses of UV (254 nm) irradiation, depending on the strain, and incubated at 37° in the dark overnight. Total viable cells were determined from serially diluted unirradiated cells.

RESULTS

High-copy suppression screen: We screened for high-copy suppressors capable of compensating for the UV repair defect of a triple ssExo$^-$ mutant (Viswanathan

TABLE 1

***Escherichia coli* K-12 strains**

Strain	Genotype	Source of derivation
BT199	F$^-$λ$^-$*thi-1* Δ*(gpt-proA)62 thr-1 leuB6 kdgK51 rfbD1 ara-14 lacY1 galK2 xyl-5 mtl-1 tsx-33 supE44 rpsL31 rac*$^-$	W. Wackernagel
CAG12156	+ *uvrC279::Tn10*	C. Gross
CS85	+ *ruvC53 eda51::*Tn*10 argE3 hisG4*	R. Lloyd
HR839	+ Δ*xonA300::cat argE3 hisG4*	Razavy *et al.* (1996)
JC10287	+ Δ*(srlA-recA) argE3 hisG4*	A. J. Clark
JC5519	+ *recB21 recC22 argE3 hisG4*	A. J. Clark
RDK1445	+ *zgb-224::Tn10 serA6 argE3 hisG4*	R. Kolodner
STL113	+ *recJ284::*Tn*10 argE3 hisG4*	Viswanathan and Lovett (1998)
STL2347	+ Δ*(xseA-guaB)zff-3139::*Tn*10kan*	Viswanathan and Lovett (1998)
STL2348	+ Δ*(xseA-guaB)zff-3139::*Tn*10kan recJ284::*Tn*10*	Viswanathan and Lovett (1998)
STL2691	+ Δ*(xseA-guaB)zff-3139::*Tn*10kan recJ2052::*Tn*10kan*	Ser$^+$ transductant P1 STL113 × STL4478
STL2701	+ Δ*xonA300::cat* Δ*(xseA-guaB) zff-3139::*Tn*10kan recJ2052::*Tn*10kan*	Cm^r transductant P1 HR839 × STL2691
STL2857	+ *uvrC279::*Tn*10*	Tc^r transductant P1 CAG12156 (C. Gross) × BT199
STL4478	+ Δ*(xseA-guaB) zff-3139::*Tn*10kan serA6 zgb-224::*Tn*10*	Tc^r Ser$^-$ transductant P1 RDK1445 × STL2347

and Lovett 1998). A genomic high-copy (300–400 copies per cell) library was prepared from *xonA xseA recJ* mutant chromosomal DNA and was used to transform the triple ssExo$^-$ mutant strain. Rather than immediately performing a random analysis of individual clones for changes in UV survival, the entire population of ssExo$^-$ transformants was enriched for cells harboring plasmids that conferred increased UV survival. An iterative enrichment was performed whereby plasmids harvested from cells that survived one round of UV irradiation were used to transform the next round of cells, which were then selected in a similar manner. The success of the enrichment process was monitored by determining the fractional survival of the population after each round (Table 2). Upon successive iterations of irradiation and transformation, the fractional survival increased steadily until round six, where the survival plateaued to 120-fold above the starting survival frequency.

Individual unirradiated transformants from the sixth round were assayed for UV survival and compared to the ssExo$^-$ strain harboring the vector alone. Of 19 isolates examined, 15 showed >30-fold increases in UV survival; the remaining 4 were identical to the vector transformant. Sequence analysis was performed for 10 isolates to determine the endpoints of the inserts. Several of the isolates were identical; overall, we isolated four distinct types. The UV survival of a representative of each isolate type (pAL1-4) is shown in Table 3. From BLAST (Altschul *et al.* 1990) sequence analysis of clones using the *E. coli* genome database, all four isolates encompassed a single region of the chromosome. Comparison of the overlapping regions of the four clones

TABLE 2

Enrichment for high-copy suppressors of a RecJ$^-$ ExoI$^-$ ExoVII$^-$ strain

Round of enrichment	Fractional survival of population at 20 J/m^2
1	9×10^{-5}
2	4×10^{-4}
3	8×10^{-4}
4	7×10^{-3}
5	9×10^{-3}
6	1×10^{-2}

The fractional survival of the transformed population of STL2701 was determined after each round of irradiation. The fractional survival of round 1 represents the survival frequency of the initial genomic library transformants. UV survival values were determined from multiple measurements from a single experiment.

TABLE 3

UV survival of ssExo$^-$ strain STL2701 carrying various *rnt* plasmid isolates

Plasmid	Fractional survival at 20 J/m^2 × 10^{-2}	Fold increase
pAL1	1 (± 0.5)	40
pAL2	1 (± 0.07)	40
pAL3	1 (± 0.03)	30
pAL4	3 (± 3)	90
pAL5	0.04 (± 0.03)	1
pBSSK$^-$	0.04 (± 0.02)	=1

Plasmids pAL1–4 harbor unique *rnt*-containing genomic fragments isolated from the iterative selection screen. pAL5, derived from pAL1, contains an N-terminal frameshift in the *rnt* ORF. Strains were assayed for UV survival relative to the pBSSK$^-$ vector, and the reported values are the averages of at least two sets of independent assays. The standard deviations of fractional survival values are shown in parentheses.

showed that they all shared one gene in common, *rnt* (Figure 1), which encodes RNase T (Deutscher and Marlor 1985). Plasmid pAL1 has the smallest genomic insert (Figure 1), which encompasses only slightly more than the *rnt* coding and promoter regions (Huang and Deutscher 1992). Although pAL2 and pAL4 contain other unknown open reading frames (ORFs), o135 and o365, pAL1 does not contain these ORFs. Plasmid pAL3, in addition to *rnt*, has nearly the entire ORF for the neighboring gene in its operon, *lhr* (Reuven *et al.* 1995).

Analysis of *rnt*: To determine if *rnt* was responsible for the increased UV survival of ssExo$^-$ cells, the gene was mutated and assayed for its ability to confer additional UV survival to ssExo$^-$ cells. pAL1 was mutated by filling in the single *Age*I site (22 bp from the beginning of the ORF), introducing a frameshift early in the coding region. Transformants of the resulting plasmid, pAL5, had nearly identical survival to cells carrying the vector (Table 3). This confirms that *rnt* is responsible for the increased repair capacity seen in ssExo$^-$ mutants carrying pAL1.

Plasmids pAL1(*rnt*) and pBSSK$^-$ (vector) were transformed into wild-type and various UV-sensitive strain backgrounds and were tested for their ability to enhance UV survival (Figure 2). The UV survival curves for the RecJ$^-$ ExoI$^-$ ExoVII$^-$ transformants are shown in Figure 2B; *rnt* clearly enhances the survival of this strain, even at high dosages of irradiation. Interestingly, *rnt* is unable to achieve the same effect in a RecJ$^-$ ExoVII$^-$ strain (Figure 2B), suggesting that *rnt* compensates predominantly for ExoI in the triple ssExo$^-$ mutant. Strains deficient in other recombination functions, namely *recA* (Figure 2A), *recBC* (Figure 2C), and *ruvC* (Figure 2C), showed no additional enhancement to their UV survival when transformed with pAL1. However, *uvrA* and *uvrC* (Figure 2A) mutant strains, deficient in nucleotide excision repair, showed a dramatic increase in UV survival when transformed with the *rnt*-containing plasmid. Wild-type cells were also tested (Figure 2C) but showed no difference in UV survival when transformed with pAL1.

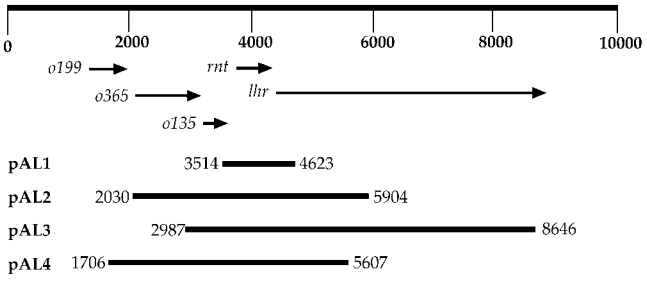

Figure 1.—Genomic insert map depicting a graphical representation of section 150 of 400 from the sequenced *E. coli* K12 genome (Blattner *et al.* 1997; GenBank accession number AE000260). Numbering of the nucleotides in this region follows the *E. coli* genome database numbering system. Five of the putative and known ORFs within the region have been included. Shown below the ORFs (with black bars) are the unique genomic DNA fragments found in plasmids pAL1–4 isolated from the iterative enrichment screen. The endpoints of each insert are given on either side of the black bars.

DISCUSSION

We have described a high-copy suppression screen using an iterative selection scheme to enrich for clones that aid UV survival in cells deficient in ExoI, ExoVII, and RecJ exonucleases (Viswanathan and Lovett 1998). Each round of selection increased the UV survival of the total population, confirming an enrichment for plasmids that confer additional UV survival. After five rounds of selection, most of the plasmids in the population contained a single gene, *rnt*, which encodes RNase T. Cells harboring *rnt* on a high-copy plasmid had ~40-fold higher UV survival frequencies at the 20 J/m^2 UV dose than vector alone, suggesting that overexpression of RNase T can increase levels of DNA repair. Disruption of the *rnt* ORF by an N-terminal frameshift mutation abolished the suppressive effect, verifying that the effect was caused by *rnt* alone. Because RNase T expression was not driven by any exogenous plasmid promoter, the suppression effect was solely caused by the endogenous *rnt* promoter expression. By previous estimates, an *rnt*-containing high-copy plasmid increases the *in vivo* levels of RNase T by 40-fold (Reuven *et al.* 1995).

E. coli RNase T was identified biochemically as an exoribonuclease. It is one of several proteins involved in the removal of the terminal adenine residue from the 3′ end of immature tRNA (Deutscher *et al.* 1985), and it is uniquely required for the removal of two 3′-terminal ribonucleotides in the processing of 5S RNA (Li and Deutscher 1995). In the absence of RNase T, 5S RNA is not processed, although this does not appear to impede its ability to function normally in translation (Li and Deutscher 1995).

It seems unlikely that an enzyme strictly involved in RNA metabolism would be capable of greatly enhancing UV survival, a phenomenon that is intimately associated with DNA damage repair. Several of our observations are more easily explained by the supposition that RNase T is also a DNase. First, the presence of *rnt* on a high-copy plasmid was able to augment the UV survival of a RecJ$^-$ ExoI$^-$ ExoVII$^-$ mutant, but not a RecJ$^-$ ExoVII$^-$ mutant, suggesting that RNase T is acting in lieu of ExoI, a 3′ to 5′ single-strand DNase with no activity on RNA (Prasher *et al.* 1983). The polarity of RNase T degradation of RNA is likewise 3′ to 5′ (Deutscher and Marlor 1985). If RNase T were to possess a 3′ degradative activity on ssDNA, its substitution for ExoI would be explained. Second, *rnt* is unable to restore any measurable amount of UV survival to *recA*, *recBC*, or *ruvC* mutants deficient in recombination functions necessary for recombinational repair, suggesting that DNA recombination is necessary for *rnt*-mediated restoration of UV survival. Although wild-type cells are also

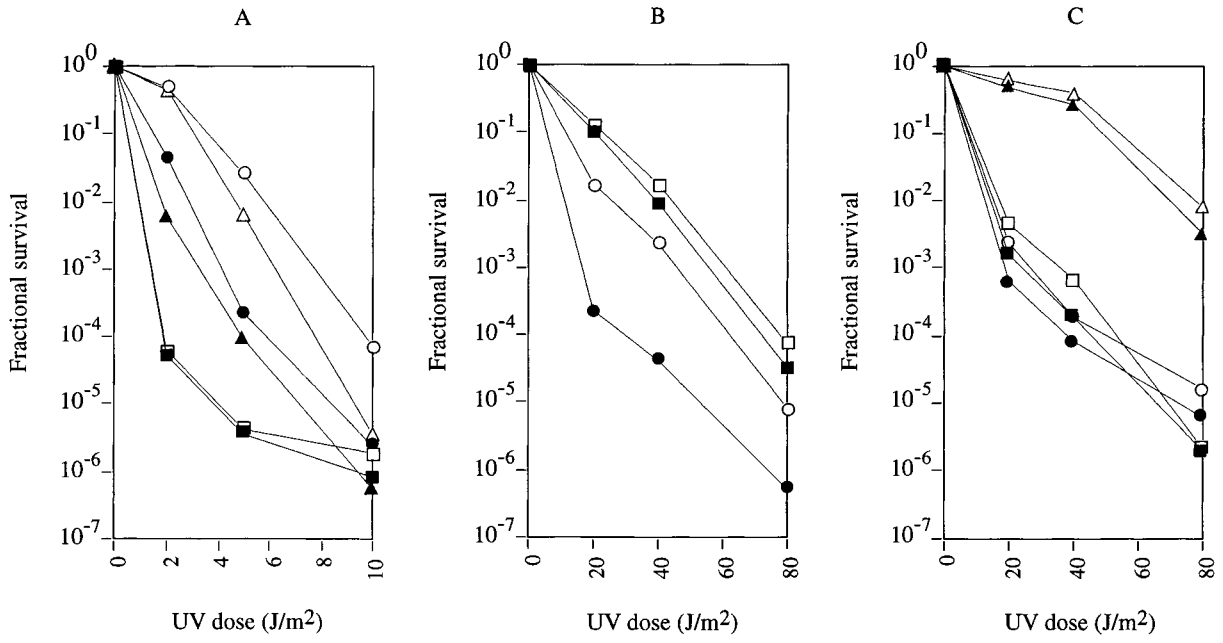

Figure 2.—UV survival of various *E. coli* mutants carrying plasmid pAL1-harboring *rnt*. Closed symbols are strains transformed with pBSSK⁻. Open symbols are strains transformed with plasmid pAL1. Survival is represented relative to the plasmid-bearing population (selected by Ap resistance); plasmid maintenance in the presence of selection was not notably affected in these strains. Survival values are means of two to four experiments. (A) *recA* (■, □), *uvrA* (▲, △), and *uvrC* (●, ○) mutants. (B) *recJ xseA xonA* (●, ○) and *recJ xseA* (■, □) mutants. (C) Wild-type (▲, △), *recBC* (■, □), and *ruvC* (●, ○) mutants.

unaffected by *rnt*, this is likely to result from the fact that in wild-type cells, the NER system and the preexisting complement of exonucleases are sufficient to perform repair such that the added benefit of *rnt* is not measurable at the UV doses tested. Third, *uvrA* and *uvrC* cells deficient in NER also benefit from high-copy expression of *rnt*. NER⁻ cells are likely inundated with lesions, so the expression of another DNA exonuclease would offer a measure of added protection, compensating for the already low levels of ssExos present in the cell. In this case, RNase T may enhance recombinational repair or provide a new pathway for excision repair. Finally, additional suggestive evidence comes from protein sequence comparisons. RNase T is a member of a new superfamily of 3′–5′ exonucleases, primarily DNases, including the proofreading domain of DNA polymerases and ExoI (Koonin 1997). The conserved motifs in this superfamily are known to comprise the active site of the 3′ exonuclease activity of DNA polymerase I (Beese and Steitz 1991; Moser *et al.* 1997), which suggests that both RNA and DNA hydrolysis can be catalyzed similarly. Our recent experiments confirm a potent single-strand-specific DNase activity is indeed associated with RNase T, and the characterization of this activity has been reported elsewhere (Viswanathan *et al.* 1998). The presence of ssDNA exonuclease activities in *E. coli*, in addition to RecJ, ExoI, and ExoVII, has been suggested previously in biochemical studies of the methyl-directed mismatch repair system (Cooper *et al.* 1993). Whether RNase T is an active participant in UV repair or other repair and/or recombination pathways remains to be investigated.

The *rnt* gene is part of a two-gene operon: its partner gene, *lhr*, was previously observed to have significant homology to ATP-dependent helicases (Reuven *et al.* 1995). It has been proposed that ssDNA exonucleases may be coupled with helicases that open up regions of double-strand DNA, exposing single-strand DNA for degradation. An example of this is the concerted action of RecJ, ExoI, or ExoVII with helicase II (UvrD) in mismatch repair reactions of *E. coli* (Cooper *et al.* 1993; Grilley *et al.* 1993). RecJ is also thought to be aided by the RecQ helicase in genetic recombination (Lovett and Kolodner 1989). It is possible that the Lhr protein may be a helicase partner to RNase T.

Our isolation of the *rnt* suppressor demonstrated the power of iterative selection *in vivo*. Iterative selection is better known from *in vitro* applications, where it forms the basis for the SELEX procedure for isolation of ligands or other functional nucleic acids (Tuerk *et al.* 1992; Gold *et al.* 1995). The advantage of our iterative approach was that plasmids that confer even incremental increases in UV survival could be isolated successfully with little quantitative screening. Moreover, we avoided the isolation of chromosomal mutations that acted as suppressors. Such an iterative approach could be broadly applied for any function associated with a quantitative genetic effect assayed by selective growth. Although it is performed most easily with a high-copy plasmid suppressor screen, in theory, one could also

enrich for chromosomal mutations if they were linked to a selectable marker (such as transposon disruption mutations) and could then be reintroduced by genetic crosses into an assay strain background. One possible disadvantage is that the scheme will tend to enrich for the best suppressors: less potent suppressors will be subsequently outcompeted. In our case, it may be useful to examine plasmids from earlier enrichment rounds, to perform the screen using a library devoid of *rnt*, or to drive expression from plasmid promoters to reveal additional suppressor genes.

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