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Bacteriophage T4 *rnh* (RNase H) Null Mutations: Effects on Spontaneous Mutation and Epistatic Interaction with *rII* Mutations

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The bacteriophage T4 *rnh* gene encodes T4 RNase H, a relative of a family of flap endonucleases. T4 *rnh* null mutations reduce burst sizes, increase sensitivity to DNA damage, and increase the frequency of acriflavin resistance (Ac^r) mutations. Because mutations in the related *Saccharomyces cerevisiae RAD27* gene display a remarkable duplication mutator phenotype, we further explored the impact of *rnh* mutations upon the mutation process. We observed that most Ac^r mutants in an rnh^+ strain contain *ac* mutations, whereas only roughly half of the Ac^r mutants detected in an *rnh*Δ strain bear *ac* mutations. In contrast to the mutational specificity displayed by most mutators, the DNA alterations of *ac* mutations arising in *rnh*Δ and rnh^+ backgrounds are indistinguishable. Thus, the increase in Ac^r mutants in an *rnh*Δ background is probably not due to a mutator effect. This conclusion is supported by the lack of increase in the frequency of *rI* mutations in an *rnh*Δ background. In a screen that detects mutations at both the *rI* locus and the much larger *rII* locus, the r frequency was severalfold lower in an *rnh*Δ background. This decrease was due to the phenotype of *rnh rII* double mutants, which display an r^+ plaque morphology but retain the characteristic inability of *rII* mutants to grow on λ lysogens. Finally, we summarize those aspects of T4 forward-mutation systems which are relevant to optimal choices for investigating quantitative and qualitative aspects of the mutation process.

The bacteriophage T4 *rnh* gene encodes Rnh, an RNase H that removes the RNA primers of DNA replication (11). This nuclease, whose structure has been determined by X-ray crystallography (21), acts as a 5′-to-3′ exonuclease on RNA-DNA and DNA-DNA duplexes and also as a flap endonuclease (2). An *rnh* null mutation (*rnh*Δ) reduces total DNA synthesis only slightly, causing instead an accumulation of short, nascent DNA fragments and reducing the burst size roughly twofold (10). *rnh*Δ is partly complemented by the host *rnhA* gene, whose impairment reduces the *rnh*Δ mutant burst size to about 10% of normal, while *rnh*Δ is more strongly complemented by the 5′-exonuclease function encoded by the host *polA* gene, whose impairment almost abolishes the *rnh*Δ mutant burst size (10). In addition to its role in DNA replication, Rnh acts in recombination repair: *rnh*Δ increases sensitivity to the lethal effects of both UV radiation and the topoisomerase inhibitor 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (*m*-AMSA), and this survival defect is epistatic to mutations in two genes required for T4 recombination repair, *uvsW* and *uvsX* (33).

T4 Rnh is related by sequence and/or structure to a family of flap endonucleases that includes examples from the archaebacteria *Methanococcus jannaschii* (13) and *Pyrococcus furiosus* (12), a eubacterium, a mammal, and the *Saccharomyces cerevisiae* DNA repair protein RAD27 (reviewed in references 26 and 30). These endonucleases remove the single-stranded flaps that can arise either as a result of displacement synthesis past the beginning of an Okazaki fragment or during genetic recombination and/or repair. However, T4 Rnh flap endonuclease is strongly inhibited by the gene-*32* single-stranded-DNA-binding protein whereas its RNase H activity is not (2). In the T4 multiprotein in vitro DNA replication system, Rnh preferentially uses its 5′-exonuclease activity rather than its flap endonuclease to remove primers and adjacent DNA from the 5′ end of lagging-strand fragments (3). A *rad27* mutation has a strong mutator phenotype at microsatellite and minisatellite sequences (14, 16) and a remarkable duplication mutator activity (31). These duplications comprised 5 to 108 bp, arose at sequences flanked by repeats of 2 to 12 bp, and were proposed to occur in a process triggered by the failure to excise DNA flaps bearing short, separated sequence repeats.

Defects in genes of T4 DNA metabolism often result in a mutator phenotype (8). Thus, T4 *rnh*Δ mutations might display mutator activity, and this activity might include a bias toward duplications. An *rnh* null mutation was reported previously to increase the frequency of T4 acriflavin-resistant (Ac^r) mutants (10). While such an increase might result from mutator activity, the relation between mutant frequency and mutation rate is often complicated and can be strikingly distorted when a mutational target (such as the *ac* gene whose knockout generates acriflavin resistance) interacts with a gene (such as *rnh*) whose product is involved in DNA metabolism. Acriflavin itself is both a topoisomerase inhibitor (19, 24) and a powerful mutagen (22). Because of the unknown impact of these potential interactions and also in order to determine whether a T4 *rnh* mutator activity had a duplication bias, we decided that the increase in Ac^r mutant frequency in an rnh^- background should be examined in some detail. The unexpectedly complex results of this investigation are described here.

MATERIALS AND METHODS

Strains. The translated portion of the *rnh* gene comprises 915 bp. The *rnh*Δ(10–777) mutation, obtained from Ken Kreuzer, is an in-frame deletion of codon-encoding bases 10 through 777 which leaves intact a middle-mode promoter near the end of *rnh* and is thus unlikely to affect downstream genes (33). The *rnh*Δ(352–847) mutation (renamed from the original) (10) removes this middle-mode promoter; in addition, because it disrupts the reading frame, it

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retains the first 117 *rnh* codons and then adds VAKFIHIL before encountering an ochre stop codon. The *rI*, *rII*, and *rV* mutants are from the Drake collection; *rIIUV58* is hyperrevertible by proflavin (5). The *rnh* mutants are in a T4D background, and the *r* mutants are from the Drake collection in a T4B background.

The *Escherichia coli* strains are from the Drake collection. B cells are su^-, and tight mutations of all the major T4 *r* loci produce the r plaque morphology (large, sharp-edged plaques) on B cells. B40 suI^+ cells are B cells containing an amber-suppressing allele. BB cells are su^-; *rII* mutants produce an r^+ plaque morphology on BB cells. KB cells are K-12(λ) and restrict the growth of T4 *rII* mutants.

Media and growth conditions. Luria-Bertani (LB) medium and Drake agars (4) were used throughout at 37°C. Stocks were grown with BB cells.

Scoring *rnh*Δ alleles. Individual plaques were resuspended in 40 μl of water, and the *rnh* gene in each was amplified by PCR. A 1,034-bp sequence (including primers) was amplified from −99 through 935 where the AUG initiation codon begins with bp 1. The upstream primer was 5′-TGAAAACACAATAGGAGC CCG-3′ (−99 through −79), and the downstream primer was 5′-TTTAGCCA TTATTCACCTC-3′ (917 through 935). The PCR consisted of 25 cycles of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C, and a final extension time of 10 min at 72°C with Display *Taq* polymerase (Display System Biotech). PCR products were separated on a 1.2% agarose gel. The *rnh*Δ(10–777) mutation produces a product of 266 bp; the *rnh*Δ(352–847) mutation produces a product of 538 bp.

Screening and sequencing Ac^r mutants. T4 stocks were plated on BB cells with acriflavin neutral (Sigma) at 0.2 μg/ml in the top agar and 0.5 μg/ml in the bottom agar to select Ac^r mutants and on unsupplemented agars to score total phages when an Ac^r mutant frequency was desired; the plates were incubated overnight. To obtain Ac^r mutants of independent origin, individual acriflavin-sensitive plaques were isolated and a single Ac^r mutant was isolated from each; the rnh^+ background was T4B.

The sequence of the *ac* gene (formerly open reading frame 52.2) (32) comprises 156 bp encoding 51 amino acids plus an ochre termination codon and extending from T4 genomic coordinate 165493 through 165338 (17). To determine their *ac* sequences, each mutant plaque was resuspended in 40 μl of water and its *ac* gene was PCR amplified with the upstream primer 5′-TCGAAGAA ATGAACCGTATGT-3′ (complementary to 165618 through 165638) and the downstream primer 5′-CTACCAATAAAGCAGCAAGGG-3′ (complementary to 165294 through 165314). The PCR consisted of 25 cycles of 1 min at 94°C, 1 min at 50°C, 1 min at 72°C, and a final extension time of 10 min at 72°C with Display *Taq* polymerase. PCR products were purified with the Qiagen purification kit. Sequencing was performed with an ABI Prizm 377 automatic sequencer with the above upstream PCR primer and the dRhodamine terminator cycle sequencing kit (PE Applied Biosystems).

Test for acriflavin mutagenicity. Working under yellow light to avoid photodynamic effects, we used *rIIUV58* in an rnh^+ or an *rnh*Δ(352–847) background to infect concentrated log-phase BB cells at 5×10^8/ml at a multiplicity of infection of 5 in LB broth at 37°C on a rotary shaker. At $t = 6$ min after infection, another multiplicity of infection of 5 was applied. At $t = 10$ min, acriflavin was added to 0 or 1 μg/ml. At $t = 30$ min, the mixture was diluted 80-fold in LB broth, and incubation was continued. Phage development was terminated at $t = 90$ min by adding chloroform. $rIIUV58^+$ revertant frequencies were determined by plating phages on KB cells (on which only rII^+ phages grow) and on BB cells (on which all phages grow).

Screening r mutants. When T4 is plated on B cells, large, sharp-edged plaque morphology mutants can be observed. These are called r mutants for rapid growth, although the number of phage particles per plaque is reduced. The r phenotype results from loss of lysis inhibition, and *r* mutations can arise at any of several loci. About 70% of spontaneous r mutants contain mutations at the *rII* locus; about 25% contain mutations at the *rI* locus; and the remainder contain *rIII*, *rV*, unmapped, or leaky or rapidly reverting *rII* mutations. Although the *rII* locus is about 10-fold larger than the *rI* locus, *rII* missense mutations are poorly detected whereas *rI* missense mutations are better detected, so that the observed ratio of *rII* to *rI* mutants is only about threefold. A typical median r mutant frequency scored by plating on B cells is roughly 6×10^{-4} in a high-titer stock grown in BB cells but is twofold to threefold lower in a resuspended plaque. We picked and restreaked all r and ambiguous plaques to verify their phenotypes.

rII mutants produce the r^+ plaque morphology on BB cells. Thus, when T4 is plated on BB cells, most of the r mutants contain *rI* mutations. A typical median r mutant frequency in a resuspended plaque plated on BB cells is roughly 2×10^{-5}.

Details of plating procedures for detecting r mutants have been described elsewhere (4). Because of the clonal nature of the distribution of mutant frequencies among stocks, the best measure of a mutant frequency is the median rather than the mean; the mean is excessively sensitive to the small proportion of stocks with high mutant frequencies, particularly jackpots. Medians from five stocks are about 95% reproducible to within twofold (25), and those from seven stocks are about 95% reproducible to within about 1.5-fold. When stocks contain similar numbers of T4 particles, ratios of mutation rates are the same as ratios of mutant frequencies. For simplicity, therefore, we present frequencies rather than rates.

RESULTS

Mutation to Ac^r. We wished first to redetermine Ac^r mutant frequencies in the wild-type and *rnh*Δ backgrounds and then to characterize the mutations at the level of DNA sequence. The Ac^r mutant frequency was previously estimated to increase 10-fold (from about 4×10^{-6}) in an *rnh*Δ(352–847) background compared to an rnh^+ background (10). Using the treatment protocol described in reference 10 but substituting BB cells for B cells, we observed a wide range (2×10^{-6} to 20×10^{-6}) of median Ac^r frequencies in several experiments in an rnh^+ background; in particular, we were plagued by a variable background of tiny plaques on the selection plates. Our Ac^r frequencies were 5×10^{-6} to 37×10^{-6} in *rnh*Δ(10–777) and 1×10^{-6} to 50×10^{-6} in *rnh*Δ(352–847). Ratios of Ac^r mutants in *rnh*Δ versus rnh^+ were 0.5 to 20 for *rnh*Δ(10–777) and 0.5 to 10 for *rnh*Δ(352–847).

In order to identify possible changes in the rates of specific kinds of Ac^r mutations, we turned to DNA sequencing. We plated wild-type and (both) *rnh*Δ strains in the absence of acriflavin, suspended well-separated plaques (of large to small rather than pinpoint size), replated these low-titer stocks in the presence of acriflavin, picked a single Ac^r mutant from each stock, PCR enriched the *ac* DNA, and sequenced. [We analyzed similar numbers of Ac^r mutants in *rnh*Δ(10–777) and *rnh*Δ(352–847) backgrounds and discerned no differences between the two backgrounds in all that follows.] T4-infected cells take up acridines at an increased rate compared to uninfected cells, and *ac* mutations arise in a gene whose inactivation prevents this uptake (27). The fractions of *ac* mutants among Ac^r mutants were different in the rnh^+ and *rnh*Δ backgrounds (Table 1). In the wild-type background, 94% of Ac^r mutants contained *ac* mutations, a result indistinguishable from the 68 of 76 mutants (89%) reported previously (32). In the *rnh*Δ background, however, only 57% of Ac^r mutants contained *ac* mutations. The locations and Ac sensitivities of the non-*ac* mutations were not investigated and remain unknown, but our primers amplify 125 nucleotides before the start codon and 23 nucleotides after the termination codon, so that the observed acriflavin resistance is unlikely to result from a defect in *ac* function; similar mutations described previously arose in a small region linked to *ac* (32). Our result suggests that roughly half of the previously described increase in Ac^r mutants in an *rnh*Δ background (10) has nothing to do with mutation at the *ac* locus.

The two *ac* mutational spectra are shown in Fig. 1 (which does not display several large mutations that are described in the figure legend). None of the mutations are complex (that is, mixtures of two or more base pair substitutions, insertions, and/or deletions). These spectra do not approach saturation, so that many of the smaller differences tend to be uninformative. However, the spectra are notable for the several sites and regions sporting multiple mutations in both rnh^+ and *rnh*Δ backgrounds. In addition, there is one apparent difference between mutation frequencies in the two *rnh* backgrounds at position 108 (three additions and four deletions in rnh^+ versus

TABLE 1. Characteristics of Ac^r mutants[a]

Ac^r mutant	rnh^+	*rnh*Δ
Total no.	65	53
ac	61	30
ac^+	4	23

[a] The *rnh*Δ column sums the values for both null alleles. For ac^+ versus *ac* in rnh^+ versus *rnh*Δ, df = 1, $\chi^2 = 23$, and $P \simeq 10^{-6}$.

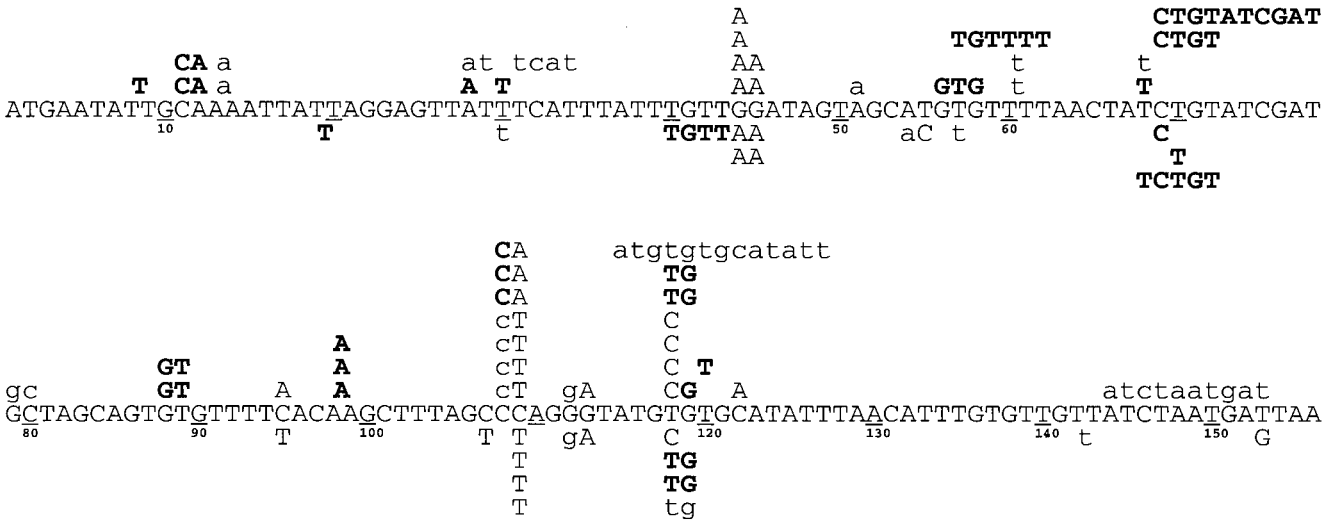

FIG. 1. Spectra of spontaneous *ac* mutations in *rnh*$^+$ (above sequence) and *rnh*Δ (below sequence) genetic backgrounds. Capital letters indicate base pair substitutions, boldface indicates insertions, and lowercase letters indicate deletions. The sequence is the *ac* gene from ATG (positions 1 through 3) encoding the initiation codon through TAA (positions 153 to 156) encoding the ochre termination codon; every 10th base is underlined and numbered beneath when possible. When an insertion or deletion could have arisen between two or more short repeated sequences, the mutation is placed in the middle of the wild-type repeats. Two or more adjacent added or deleted bases represent a single mutation, and repeated mutations are stacked. There are no complex mutations containing mixtures of base pair substitutions, insertions, and/or deletions. Note that several mutations are not shown: deletions of 32 bases between TTTT at 59 through 62 and at 91 through 94 that occurred three times in *rnh*$^+$ and twice in *rnh*Δ and one addition of 99 bases that occurred once in *rnh*Δ (see text).

no mutations in *rnh*Δ). A notable observation of multiple differences with little change in total mutation rate has been described elsewhere for differing gene-*43* backgrounds (32). A much larger collection of *ac* mutations would have to be characterized to determine whether any of the differences in the two *rnh* backgrounds are real.

The classes of mutations are summarized in Table 2. In contrast to Table 1, there is no significant difference between the frequencies of the various kinds of mutations recovered from the two genetic backgrounds; when comparisons were made between the 11 categories of observed base pair substitutions and additions and deletions of base pairs, df = 10, χ^2 = 10.5, and P = 0.40. Few mutator mutations produce mutations identical to those arising in the nonmutator background. It therefore seems likely that the previously reported increase in Ac^r mutants in an *rnh*Δ background (10) was due at least in part to the non-*ac* mutants that are specifically selected in that background and perhaps to other less well characterized factors.

Given the duplication propensity of the yeast *rad27* mutator mentioned earlier, it is notable that the proportion of duplication mutations in the two sets of *ac* mutations is virtually identical (10 of 61 in the *rnh*$^+$ background and 5 of 30 in the *rnh*Δ background). These duplications are shorter than most of those observed in the yeast *rad27* mutator: the T4 duplications included 12 of 1 bp, 6 of 2 bp, and 1 each of 3, 4, 6, and 10 bp in the *rnh*$^+$ background and 3 of 1 bp, 2 of 2 bp, and 1 each of 4, 5, and 99 bp in the *rnh*Δ background. All of the addition mutations were tandem duplications. These can be of two general types: those in which no flanking sequence repeats existed in the wild-type sequence and those apparently arising from repeats. The latter can be described as RiR→ RiRiR where R is a repeated sequence (which can be as short as a single base pair) and i is an intervening sequence between the repeats. Such mutations can arise by slipped mispairing (29) and are characteristic of the yeast *rad27* duplication mutations (31). Of the 22 insertions in the *rnh*$^+$ background, 12 have slippage-like structure and are additions of only 1 or 2 bases. Of the eight insertions in the *rnh*Δ background, three have slippage-like structure, three are additions of only 1 or 2 bases, and one is an addition of 99 bases where R is CATTT at positions 32 through 36 and 131 through 135. Only this single

TABLE 2. Kinds of *ac* mutations arising in different *rnh* backgrounds

Mutation	No. of mutations in background:	
	rnh$^+$	*rnh*Δ
Total no. of *ac* mutations	61	30
T→C	4	2
T→G	0	1
G→A	7	5
C→T	4	6
C→A	5	0
A:T→G:C	4	2
A:T→C:G	0	1
G:C→A:T	11	11
G:C→T:A	5	0
Transition	15	13
Transversion	5	1
Missense	11	6
Nonsense	9	8
+A:T	8	2
+G:C	4	1
+≥2 bp	10	5
−A:T	6	4
−G:C	5	1
−≥2 bp	8	3
Addition	22	8
Deletion	19	8

TABLE 3. Effect of *rnh*Δ on acriflavin-induced mutation

rnh allele	Acriflavin (μg/ml)	Expt no.	No. of $r^+/10^8$		RM[b]	RBS[c]
			Total	Induced[a]		
+	0	1	17			
		2	36			
		3	31			1.00
	1	1	77	60		
		2	273	237		
		3	70	39		0.26
Δ(352–847)	0	1	16			
		2	45			
		3	43			1.00
	1	1	187	171	2.9	
		2	315	269	1.1	
		3	137	95	2.4	0.15

[a] Net mutagenesis for acriflavin at 1 μg/ml compared to 0 μg/ml (for instance, in experiment 1, 77 − 17 = 60 and 187 − 16 = 171).
[b] Relative net mutagenesis in *rnh*Δ compared to rnh^+ (for instance, in experiment 1, 171/60 = 2.9).
[c] Relative to the mean of the three burst sizes compared to values for no acriflavin, for which the mean burst sizes were 409.6 for rnh^+ and 408.0 for *rnh*Δ (a coincidental near-identity for a value that typically shows considerable variation).

mutation of the 30 additions resembles the duplications arising in the yeast *rad27* mutator.

Tests for acriflavin mutagenicity in the screen for Ac^r mutants. Acriflavin is a mixture of 3,6-diamino-10-methylacridinium chloride and 3,6-diaminoacridine (proflavin), both of which are strong frameshift mutagens when present during T4 replication (22). Thus, the acriflavin test might be intrinsically mutagenic. Furthermore, because acridines preferentially mutate and promote breakage at topoisomerase sites in T4 DNA (19, 24), and because an *rnh* mutation may interfere with the repair of such breakage (33), the acriflavin test may preferentially kill or mutate replicating genomes carrying an *rnh* defect.

This possibility was tested by comparing acriflavin-induced reversion of *rIIUV58* in *rnh*Δ and rnh^+ backgrounds (see Materials and Methods). *rIIUV58* was chosen because its reversion is unusually strongly promoted by proflavin mutagenesis (5). The results of three experiments appear in Table 3. The variation is typical of experiments involving mutagenesis with acridines but the trend is clear: acriflavin (at a concentration twice that used in our measurements of Ac^r frequencies, but for a shorter time) is slightly more mutagenic (2.1-fold on average) in an Δ*rnh* background than in an rnh^+ background. Although *rnh* mutations typically reduce burst sizes, no reduction was seen in these particular experiments. Proflavin treatments also typically reduce burst sizes, and the reduction was slightly greater (85 versus 74%) in the *rnh*Δ background. The small difference in relative net mutagenesis observed with this very sensitive target suggests that the Ac test is not significantly more mutagenic to *rnh*Δ than to rnh^+ strains. This surmise is supported by the lack of any increase in the frequency of base pair additions or deletions among *ac* mutants in the *rnh*Δ background compared to the rnh^+ background (Table 2).

Absence of an *rnh* effect on mutation from r^+ to *rI*. Most r mutants detected on *E. coli* BB cells bear *rI* mutations. The *rI*-encoded protein appears to sense superinfection and to carry out the first step in establishing lysis inhibition (23) but is not obviously involved in DNA metabolism. The 97-codon *rI* locus is large enough to fairly sample average mutation rates. Therefore, in order to determine whether an *rnh*Δ mutation affects rates of spontaneous mutation in a gene other than *ac*, we scored frequencies of r mutants on BB cells. The results

TABLE 4. Effect of *rnh*Δ on *rI* mutant frequencies

rnh allele	No. of stocks	Median no. of plaques/10^5	
		r	r + sr
+	7	6.6	6.6
Δ(352–847)	7	5.2	7.4

appear in Table 4. Two entries per *rnh* genotype appear for mutant frequencies: values for sharp-edged plaques displaying the classical r phenotype and values also including slightly fuzzy-edged plaques (sr, for "semi-r") which we anticipated would be caused by leaky missense mutants. To confirm this conjecture, we isolated eight such sr plaques and sequenced them; four contained *rI* missense mutations, one contained a frameshift mutation near the end of the gene, and three contained no *rI* mutation. Therefore, the values in the "r + sr" column were computed by multiplying the sr contribution by five-eighths. Mutant frequencies such as those in Table 4 based on seven stocks are usually reproducible to about 1.5-fold (25). The data in Table 4 reveal no *rnh*Δ mutator activity on the *rI* gene.

Epistasis between *rnh* and *rII* mutations. Before undertaking the experiments detailed in Tables 3 and 4, we sought to determine the frequencies of r plaque morphology mutants in stocks plated on *E. coli* B cells. On B cells, roughly 70% of spontaneous r mutants carry typical *rII* mutations; about 25% carry *rI* mutations; and the remainder carry leaky or rapidly reverting *rII* mutations, *rIII* mutations, or *r* mutations at other less well characterized loci. Because the *rII* locus comprises about 3,135 bp, the frequency of r mutants is usually much higher on B cells than on BB cells, even though missense mutations are more efficiently detected in *rI* than in *rII*. Thus, an r-mutant screen on B cells was expected to be a simple, sensitive test for mutator activity in *rnh*Δ mutants. The results appear in Table 5. Contrary to our expectations, frequencies of r mutants are clearly reduced severalfold in a *rnh*Δ background. (The data suggest that this effect may differ in magnitude between the two different *rnh*Δ alleles, but this possibility was not further explored.)

Because our results with the *ac* and *rI* systems gave no hint of an antimutator effect, we suspected that the reduced frequencies of r mutants in Table 5 reflected some special property of *rnh rII* double mutants such as synthetic lethality or epistasis. This suspicion was supported by the results of crosses of the form *rnh*Δ × *r* where *r* = *rII* versus *rI* or *rV*. If recombinant *rnh*Δ *r* double mutants were lethal or indetectable, then the frequency of r mutants should fall in the cross progeny compared to the parental mix. We observed no change in *r* frequencies in *rnh*Δ crosses against *rI* or *rV* (average progeny/parental ratio = 1.02) but a marked decrease in *rnh*Δ crosses against *rII* mutants (average progeny/parental ratio = 0.48) with both *rnh*Δ mutations. In addition, because the *rnh*Δ allele causes small plaques, we collected numerous progeny with a

TABLE 5. Effect of *rnh*Δ on r mutant frequencies scored on *E. coli* B cells

rnh allele	No. of stocks	Median r/10^4	
		Expt 1	Expt 2
+	7	7.4	9.3
Δ(352–847)	7	4.5	
Δ(10–777)	7	1.8	2.7

small-r (sharp-edged) phenotype and tested them for the *rnh*Δ allele by PCR; we found none.

We next performed crosses between *rnh*Δ mutants and five different *rII* amber mutants with B40 *suI*$^+$ host cells for the cross and for plating the progeny (so that all progeny necessarily displayed the r$^+$ plaque morphology). Ten small-plaque progeny were isolated from each cross. These were tested for inability to grow on *E. coli su*$^-$ K(λ) cells, a characteristic of most *rII* mutations; among the nongrowers, we screened for the presence of the *rnh*Δ allele by PCR. Each of the crosses readily yielded progeny that appeared to bear both mutant alleles. These progeny were backcrossed against the original *rII* parent to confirm the identity of the *rII* allele. Thus, *rII* mutations (including the frameshift mutation *rIIUV58* described previously) display an r$^+$ plaque morphology in the presence of an *rnh* null allele but do not regain the ability to grow on K(λ) cells.

DISCUSSION

Apparent lack of an *rnh*Δ effect on mutation rates. The evidence as a whole indicates that bacteriophage T4 *rnh* null mutations do not affect mutation rates to an appreciable extent. No change was detected with the *rI* system, and the increase in mutant frequency reported previously with the Ac^r system is unlikely to reflect a corresponding increase in mutation rate for three reasons. First, we did not find a reliable increase in the frequency of Ac^r mutants by the reference protocol (10), although an alternative protocol (32) appears to be more robust. (We used a different strain of B cells, namely, BB or B Berkeley, but we have no reason to suspect that B and BB would behave differently in *rnh*Δ phage infections.) Second, while most Ac^r mutants in an *rnh*$^+$ background contain *ac* mutations, many in an *rnh*Δ background do not. Third, there was no discernible difference in the kinds of *ac* mutants produced in the two backgrounds, whereas most mutator mutants display specificity. Therefore, either the host DNA polymerase I 5′-exonuclease activity that moderately complements the loss of Rnh or the host RNase H that weakly complements the loss (10) seems to provide any fidelity components that might be missing in an *rnh* mutant.

Note, however, that changes in mutational specificity can occur without changes in mutation rate (6, 32). The data in Table 2 can exclude only major changes in mutational specificity. However, they do appear to exclude any strong slippage-like duplication bias in an *rnh*Δ background.

Epistasis between *rnh* and *rII* mutations. Just as a mutator effect was shown to be an unlikely explanation for an increased Ac^r mutant frequency in an *rnh*Δ background, an antimutator effect was shown not to be the cause of a decreased r mutant frequency in an *rnh*Δ background. Instead, the severalfold decrease in the median r mutant frequency in stocks plated on B cells reflected the unanticipated r$^+$ plaque morphology of *rII rnh*Δ double mutants, although such double mutants retain the typical inability of *rII* mutants to grow on a λ lysogen.

The *rII* locus interacts with several other loci important in DNA metabolism (20, 23, 28). *rII* mutations produce the r$^+$ plaque morphology when accompanied by a *49tsC9* (Holliday resolvase) mutation or by the gene-*32* (single-stranded-DNA-binding protein) mutation *32tsL171*. Conversely, *rII* mutations partly suppress *32tsL171* and also suppress gene-*30* (DNA ligase) mutations. Thus, *rII* function is extensively connected to DNA metabolism, albeit in poorly understood ways. Indeed, the two *rII*-encoded proteins not only are associated with the cell membrane but also cosediment with the huge multiprotein DNA replication complex (9). The failure of *rII* mutations to express the r plaque morphology on BB and K-12 host cells suggests that the *rII* locus has only an indirect role in lysis inhibition (23). However, this pattern of interactions with DNA metabolism highlights the need for caution when using the *rII* system as a mutational reporter gene (7, 8).

TABLE 6. T4 forward-mutation systems

Target trait	*rII* system	*ac* system	*rI* system
Target size (bp)[a]	3,131	158	311
Quantitation[b]	Easy	Difficult	Low background
Collection of independent mutants[c]	Easy	Easiest	Can be difficult
Major hot spots[d]	50%	Absent	25%
Missense detection[e]	Poor	Good	Good
Involvement in DNA metabolism	Strong	Some	None

[a] The mutational target size is estimated as the number of transcribed base pairs + 20. While *ac* and *rI* and their immediate environs can be sequenced in a single lane, *rII* mutants require deletion mapping prior to sequencing.

[b] An r frequency can be estimated by visually screening 20 to 40 plates, each bearing about 800 plaques on B cells. To detect sufficient *rI* mutants on a host cell that does not reveal *rII* mutants, about 300 to 600 plates, each bearing about 800 plaques, would have to be screened. However, scoring either total r mutants or *rI* mutants obviously becomes much easier in a mutator background or after mutagenesis. The *ac* mutant frequency can be measured by plating one to four plates at low dilutions and one to two plates at a high dilution, but the results may be poorly reproducible and the smaller *ac* plaques may become difficult to see if initiated from phages bearing DNA damage or deleterious background mutations.

[c] Mutants of independent spontaneous origin are collected each from a different stock, which typically consists of a small sample of a single plaque. For *r* mutants, the amount of work simply reflects typical mutant frequencies. For *ac* mutants, a single plate suffices to select at least one Ac^r mutant.

[d] *rII* harbors three $(A:T)_6$ hot spots for spontaneous mutation that together contribute about 50% of all mutants. There are no such sequences in *ac* or *rI*, but *rI* contains one large complex hot spot. Warm spots exist in all three genes.

[e] The ratio of *rII* missense mutations to nonsense mutations appears to be very low, and many *rII* missense mutations are almost indetectable by plaque morphology (15). The data in Table 2 show that missense mutations are fairly efficiently detected in *ac*, and preliminary results indicate that missense mutations are well detected in *rI*.

The *ac* mutational spectrum. Because our spectra are based on only 91 mutations distributed among many sites and classes, it is premature to analyze the *ac* mutational spectrum in detail. In any case, this is not the purpose of the present report, and we leave such analysis to our colleague and *ac* champion Lynn Ripley (32). Note, however, that the pooled North Carolina and *43*$^+$ New Jersey *ac* mutations may differ. The two sets were obtained by somewhat different selection procedures that may have affected the efficiency of recovering missense mutations, which comprised 17 of 34 of the North Carolina base pair substitutions but 27 of 33 of the New Jersey base pair substitutions. The distribution of mutations among classes may be different in the two collections (df = 13, $\chi^2 = 22.4$, $P = 0.050$), but if they are, much of the difference is due to five G→C (Gly→Arg) mutations at position 112 that are unique to the New Jersey collection; if those five mutations are not considered, df = 12, $\chi^2 = 15.7$, and $P = 0.21$.

Optimal T4 targets for forward mutation. The sensitivity of a mutation test reflects the magnitude of changes produced by physical or chemical treatments or genetic modifiers. The specificity of a mutation test reflects the information that it provides about what kinds of mutations arise and where. Reversion tests tend to maximize sensitivity and specificity but may provide uncharacteristic responses conditioned by the local DNA sequence in which the reverting base pairs reside; some potential responses may escape detection altogether, and little or no information is generated concerning the effects of local DNA sequence. In contrast, forward-mutation tests may display reduced sensitivity because only certain kinds of mutations tend to be increased experimentally, but such tests may

display high sensitivity with regard to effects of local sequence on mutation. Currently, there are three useful T4 targets for forward mutation, *rII*, *ac*, and *rI*, each with its particular advantages and disadvantages (Table 6).

Until recently, the most frequently used T4 mutation system employed the powerful *rII* locus, which can be used to measure both forward mutation and reversion. A large amount of information is available about both spontaneous and induced mutation in the *rII* locus. However, this system has several drawbacks. One is lack of information about the biochemical roles of the *rII*-encoded proteins (reviewed in reference 28 and largely unchanged since then), including their equivocal role in lysis inhibition (23). Another is the involvement of *rII* function in DNA metabolism, as detailed above. A third is the large size of *rII*, so that very large numbers of mutants must be isolated when it is desirable to identify preferentially mutable sites, and mutations must be mapped before they can be sequenced efficiently. A fourth is the inefficiency with which missense mutations are detected.

The *ac* and *rI* systems share the advantages of small size and relatively efficient detection of missense mutations (1, 23, 32). (We will describe several aspects of the *rI* system in more detail in a later article.) Small size has two advantages, ease of sequencing and ease of detecting differential mutability from site to site. The *ac* system is the easiest for collecting mutants of independent origin but may be difficult to quantitate, and the Ac^r screen may perturb DNA metabolism. The *rI* system seems to be totally independent of DNA metabolism (although crucial for lysis inhibition, which leads to more, apparently quite normal DNA synthesis). The main drawback of the *rI* system is the low frequency of spontaneous mutants, which renders quantitation and mutant collection tedious; however, this problem disappears under conditions that increase mutant frequencies by 10-fold or more.

We also investigated the *alc* system, in which forward mutations can be selected by plating on a specific host (18). Unfortunately, the resulting *alc* plaques are small under a variety of plating conditions, so that quantitation is difficult. In addition, *alc* mutations cause T4 DNA to contain ordinary cytosine instead of 5-hydroxymethylcytosine, a difference that perturbs DNA metabolism.

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