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Ligase-Defective Bacteriophage T4

I. Effects on Mutation Rates

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Temperature-sensitive mutations in bacteriophage T4 gene 30 (polynucleotide ligase) were examined for their effects on spontaneous and proflavine-induced frameshift mutagenesis in the *rII* and *ac* (acridine resistance) cistrons. Only small (fourfold or less) effects on mutation rates were observed, even when selection artifacts involving suppression of gene 30 mutations by *rII* mutations were taken into account. The deoxyribonucleic acid ligase gene of T4 therefore appears to be only a minor determinant of frameshift mutation rates. This result is consistent with the particular nature of frameshift mutagenesis in bacteriophage T4.

A fruitful current method for analyzing mutational mechanisms is to test for effects on mutation rates brought about by defects in genes which encode various enzymes affecting deoxyribonucleic acid (DNA) metabolism. This approach is particularly inviting for the study of frameshift mutagenesis, since the theory of frameshift mutagenesis invokes the action of several specific enzymes of DNA degradation and resynthesis (17). Mutant alleles of several genes affecting DNA metabolism have recently been reported (4) to enhance the reversion rates of bacteriophage T4*rII* frameshift mutations, but strong effects were observed only with mutations in genes 32 (Alberts protein) and 43 (DNA polymerase).

Gene 30 of bacteriophage T4 produces a polynucleotide ligase (10) which catalyzes the final step in the repair of breaks in phosphodiester bonds in double-stranded DNA. Mutants of gene 30 can initiate but cannot long continue DNA synthesis, and both parental and progeny DNA strands accumulate single-strand interruptions (15). The general theory of frameshift mutagenesis (17) hypothesizes that ligase action constitutes the final step in fixation of the mutational lesion into a mutational heterozygote. A mutant ligase might therefore alter the frameshift mutation rate, that is, act as a mutator mutation. Little or no increase was observed previously in the spontaneous reversion of two *rII* frameshift mutations when coupled with either of two temperature-sensitive mutations in gene 30 (4). An earlier report (16), on the other hand, suggested that one of these *ts* ligase mutations did increase the proflavine-induced reversion rate of an *rII* frameshift mutation.

Although the T4*rII* system is usually excellent for measuring mutation rates, it has an important shortcoming for studies involving gene 30: *rII* mutations suppress the lethality of gene 30 mutations (3, 13). T4*rII* mutations apparently reduce the amount of ligase needed for phage growth, either directly or indirectly decreasing endonuclease activity or else freeing host ligase for action on phage DNA (11, 12). An *rII*$^+$ revertant of a gene 30-*rII* double mutant should therefore be at a selective disadvantage compared to the double mutant, because the revertant no longer suppresses the ligase defect. Could this selection influence the measurement of reversion frequencies, thus masking mutator or mimicking antimutator activities? The studies presented here reconsider the effect of a ligase defect on frameshift mutation rates in the light of this *rII*-gene 30 interaction.

MATERIALS AND METHODS

Phage and bacteria. The T4*rII* frameshift mutants *rUV6* and *rUV58* are from the Drake collection (5), and the frameshift mutants *rFC7*, *rFC40*, and *rFC55* are from the Crick collection (2). The gene 30 mutants *tsA80*, *tsB20*, and *tsN7* are from the Caltech collection (9). *Escherichia coli* BB and B are permissive hosts for *rII* mutants. *E. coli* KB, a K-12(λ) strain, is nonpermissive for *rII* mutants. N1325 is a nonlysogenic derivative of strain N1252, which overproduces a partially defective host ligase; N1325 and its parent N953 were obtained from M. Gellert (11).

Construction of double mutants. It was frequently necessary to construct gene 30 *ts*-gene *rII* double mutants by recombination. Both characters are normally easily scored by spot tests: the *ts* mutants fail to grow at 42 C, and the *rII* mutants fail

to grow on lambda lysogens. An *rII* mutation, however, suppresses the lethality of a gene 30 mutation. Nevertheless, we found that the suppression of gene 30 *ts* mutants was sufficiently incomplete at 43 C to permit scoring of double mutants. As a further proof of structure, the double mutants were usually split to recover their two components.

Measurement of mutant frequencies. General methods for handling bacteriophage T4 were described (1). L broth was used throughout, except where indicated. Multiple cycle stocks were initiated with BB cells at approximately 10^7 per ml and with phage at a multiplicity of infection of 0.005, and were aerated on a rotary shaker. Chloroform was added to complete lysis after 6 hr at 37 C or 10 hr at lower temperatures. Several (three to six) stocks were grown in parallel to determine each revertant frequency, and rII^+ revertant frequencies were determined by plating on KB and BB cells, with incubation at 25 C. Total phage particles per KB plate were always kept below 10^8 to avoid crowding effects. The revertant frequencies cited are the lowest obtained in each series; this method minimizes the effects of overestimation because of clonal effects in the appearance of mutants in growing populations, and is at least as accurate as taking average or median values, since stocks with anomalously low revertant frequencies are very unlikely to arise for stochastic reasons (6). At least in the present experiments, using the lowest frequency provides the same relationships as does using the median frequency. When reconstruction controls were performed, they were always run in parallel with the measurement to be controlled. The reconstruction controls were initiated with *rII* mutants to which revertants had been added to a frequency of 0.1%, a level far above the spontaneous or induced revertant frequency.

Acridine-resistant mutants were screened by plating on plates poured with 35 ml of bottom agar containing 0.5 μg of acriflavine neutral per ml, and also adding 9.4 μg of acriflavine neutral to the 2.5-ml top agar.

Proflavine mutagenesis. Log-phase BB cells at 37 to 38 C in L broth adjusted to *p*H 7.8 were infected with phage at a multiplicity of infection of 8. At 8 min, proflavine was added to 8 μg/ml. At 16 min, another phage multiplicity of infection of 8 was added to induce lysis inhibition. At 40 min, the complexes were diluted 50-fold into L broth. Lysis was completed with chloroform at 70 min, and the progeny were assayed immediately. In some experiments, however, the conditions of Sarabhai and Lamfrom (16) were employed.

RESULTS

Spontaneous reversion of rII mutants. In previous experiments designed to search for T4 ligase mutator activities (4), reversion rates of *rII* frameshift mutants were compared in wild-type and gene 30 *ts* background. We also performed a number of such measurements (Table 1), and we too failed to observe any significant mutator effects. Because *rII* mutations suppress ligase defects in T4, however, and because rII^+ is dominant over *rII* in suppression, complexes containing rII^+ revertants in a gene 30 *ts* background should often produce smaller burst sizes than would complexes containing only *rII* mutant genomes. Such selection could easily mask any mutator activity arising from the gene 30 *ts* mutation, even though the experiments of Table 1 were performed at 32 C to reduce the lethal effects of the gene 30 *ts* mutation.

This difficulty can be partly overcome by performing reconstruction selection controls in which stocks are grown from mixtures of gene 30 ts-rII^+ and *ts-rII* particles; the initial mixture contains the rII^+ component at a frequency very much higher than would result from reversion, typically about 0.1%. The average selection coefficient per replication s is then determined from the relationship $f/f_0 = (1 - s)^i$ (i) where f is the final rII^+ frequency, f_0 is the initial rII^+ frequency, and i is the number of genome doublings occurring during growth, determined from the initial and final titers. The mutation rate per replication m can then be estimated from a simple modification of a standard relationship (6) between mutation rates and mutant frequencies by the solution of $dm = [m + (1 - s)M/N]dN$ (ii), in which M is the number of revertants in the population at a given moment and N is the population size at that moment. (This formulation assumes that the frameshift mutation rate is proportional to the number of replications, but does not necessarily imply that frameshift mutagenesis is a

TABLE 1. *Spontaneous rII^+ revertant frequencies in stocks grown at 32 C*

rII allele	Gene 30 allele	Revertant frequency: $rII^+ \times 10^8$	rII^+ ratio: ts/ts^+
rUV6	ts^+	78	
	tsA80	47	0.60
	tsB20	49	0.62
	tsN7	31	0.39
rUV58	ts^+	12	
	tsA80	21	1.7
	tsB20	12	0.95
	tsN7	12	0.97
rFC7	ts^+	43	
	tsA80	25	0.57
	tsB20	33	0.77
rFC40	ts^+	16	
	tsA80	27	1.6
	tsB20	38	2.3

direct consequence of DNA replication, as opposed, for instance, to concomitant DNA repair.) The solution of this equation is $m = s[f - f_0 e^{-s \ln (N/N_0)}]/[1 - e^{-s \ln (N/N_0)}]$ (iii), where N_0 and N are the initial and final population sizes and f_0 and f are the initial and final mutant (or revertant) frequencies. In practice, the starting inoculum is small enough so that $f_0 = 0$, which simplifies the use of equation iii. In cases where $s = 0$, equation iii takes on the more familiar (6) form $m = (f - f_0)/ln(N/N_0)$ (iv), which is also usually employed under conditions where $f_0 = 0$.

All measurements were made on control and experimental stocks grown in parallel, since in our hands the magnitude of s varies considerably from day to day. Furthermore, the reconstruction controls were run at least in duplicate. It is evident from equation iii that strong selection (for instance, $s = 0.3$) can cause a marked (for instance, 10-fold) increase in the *rII* reversion rate in the *ts* background to appear as an almost insignificant (twofold) increase. Equation iii will tend to overcorrect when selection is strong, because s is expressed per replication whereas selection probably actually occurs per infectious cycle. It is not immediately obvious how to correct for this discrepancy except by arithmetical simulation. We estimate that the overestimation is approximately 60% when $s = 0.4$, but is a negligible 5% when $s = 0.2$.

In the next series of experiments, both selection coefficients and revertant frequencies were measured. In addition, instead of comparing rII^+ revertant frequencies in *ts* versus wild-type backgrounds, they were compared in *ts* backgrounds in stocks grown at 26 C (where the *ts* ligase defect is probably virtually absent) and at 37 C (where the *ts* defect is substantial). This procedure eliminated any complications which might have resulted from growing and assaying *rII* stocks of differing genetic backgrounds, whether reference is made to the specific ligase *ts* marker or to other, unsuspected differences. The results appear in Table 2. Rather large differences were observed among repeated measurements with two of the combinations tested (*tsA80-rFC7* and *tsB20-rFC7*), but a distinct trend toward increased reversion rates was observed at the more nonpermissive temperature. Nevertheless, the effects were small, compared, for instance, to many of the effects observed with mutator mutations in the DNA polymerase gene 43 (7).

Proflavine-induced reversion of rII mutants. Considerable efforts were made to confirm an earlier report (16) that the gene 30 mutant *tsB20* increases the proflavine-induced reversion of the *rII* frameshift mutant *rFC55* by an order of magnitude. In similar experiments, we generally observed only small increases (Table 3). Out of 25 measurements, the strongest example of mutator activity was observed with *tsB20-rFC40*, which once yielded a 6.6-fold increase in revertant frequency in the *ts* compared to the wild-type background. A set of experiments performed under what appear to be the identical conditions of the earlier report (16) yielded only a slight increase. Since the host DNA ligase has been shown to compensate partially for the phage ligase deficiency (11), we also performed experiments by using a host cell mutant N1325 which harbors a host ligase

TABLE 2. *Spontaneous rII^+ reversion rates in stocks grown at 25 and 37 C*[a]

Mutant	Expt	25 C			37 C			Ratio: m(37 C)/m(25 C)
		f	s	m	f	s	m	
rFC40-tsA80	1	22	0.01	1.5	24	0.32	7.2	4.8
	2	17	0.03	1.3	9.4	0.38	3.6	2.7
rFC40-tsB20	1	87	0.00	7.7	81	0.16	16	2.1
	2	25	0.00	1.7	19	0.09	2.4	1.4
rFC55-tsB20	1	140	0.01	9.3	60	0.17	11	1.2
	2	150	0.01	10.6	110	0.10	14	1.3
rFC7-tsA80	1	56	−0.02	3.1	120	0.03	11	3.6
	2	180	0.00	11	62	0.02	4.9	0.45
rFC7-tsB20	1	160	0.01	12	260	0.00	19	1.6
	2	270	0.03	23	220	0.02	17	0.73

[a] f, Revertant frequency ($rII^+ \times 10^8$); s, selection coefficient (see equation 1); m, mutation rate (rII^+ per replication $\times 10^8$).

TABLE 3. *Proflavine-induced rII⁺ revertant frequencies*

rII allele	Gene 30 allele	No. of expts	Control[a]		Proflavine[a]		Net *rII⁺*	*rII⁺* ratio: *ts/ts⁺*
			rII⁺	BS	*rII⁺*	BS		
rFC7	*ts⁺*	4	2.0	610	97	190	95	
	tsA80	4	17	430	160	44	143	1.5
	ts⁺	3	1.8	540	88	170	87	
	tsB20	3	13	160	214	18	202	2.3
rFC40	*ts⁺*	4	6.6	500	45	180	38	
	tsA80	4	1.2	290	96	25	95	2.5
	ts⁺	5	4.2	570	39	140	35	
	tsB20	5	1.1	260	96	7	95	2.7
rFC55	*ts+*	3	2.1	710	45	70	43	
	tsB20	3	1.9	750	62	15	60	1.4
	ts⁺	2[b]	2.8	530	26	80	23	
	tsB20	2[b]	2.2	590	36	10	34	1.5
	ts⁺	4[c]	2.0	700	20	370	18	
	tsB20	4[c]	2.2	160	29	36	27	1.5

[a] *rII⁺* revertant frequencies are $\times 10^8$; BS, burst size.
[b] Procedure of Sarabhai and Lamfrom (16) was used.
[c] Host cell was N1325 instead of BB.

defect. The effect of *tsB20* on the reversion of *rFC55* was again slight.

Selection artifacts are very improbable in all of these proflavine experiments. The frequency of *rII⁺* revertants induced by proflavine is generally very much higher than the input of preexisting spontaneous revertants, and experiments reported in the following paper (13) show that the gene 30-*rII* interaction is complete by the time proflavine is added, so newly induced *rII⁺* revertants are unlikely to reintroduce selection against the *ts* gene 30 mutation.

Mutation to acriflavine resistance. Finally, we attempted to avoid the complexities of the *rII* system in the present context by measuring mutation in the *ac* cistron (8). Stocks of *tsA80* and T4D (wild type) were exposed to proflavine, and the frequencies of forward mutations from acridine sensitivity (*ac*) to acridine resistance (*ac^r*) were determined by plating with and without acriflavine neutral. Since measurements of *ac^r* mutant frequencies show considerable fluctuations, depending upon plating conditions, care was taken to maintain invariant assay procedures and to make all comparisons in parallel. Furthermore, since *ac^r* is recessive to *ac⁺* (8), the phage-to-cell ratio was always maintained below 0.02 on the acridine plates. In addition, small corrections were made to all revertant frequencies for efficiencies of plating, which were estimated by measurements on 10 independently isolated *ac^r* mutants from both T4D and *tsA80*. The average efficiency of plating of *tsA80* on acriflavine plates compared to standard plates was 0.83 (range 0.5 to 1.8), and the average efficiency of plating of T4D was 1.05 (range 0.6 to 1.8). The final results appear in Table 4. We observed no significant effect of the ligase defect on the proflavine-induced *ac^r* frequency.

DISCUSSION

This laboratory has been engaged recently in searching for the genetic determinants of spontaneous and induced mutation rates in bacteriophage T4. Our approach has been to study the effects on mutation rates brought about by genetic defects in those genes of DNA replication and repair whose specific functions have been identified. To date, marked effects on various types of point mutation rates have been observed from perturbations of genes 43 (DNA polymerase) (4, 7), *x* (generalized repair) (*unpublished results*), 32 (Alberts protein) (4; *unpublished results*), and 42 (deoxycytidylate hydroxymethylase) (*unpublished results*), whereas small or no effects have been observed with gene *v* (pyrimidine dimer excision) (*unpublished results*) and with a number of other genes whose function remains unclear (4). The genes which appear, in the above studies, to be important in the determination of the frameshift mutation rate are 32, 43, and *x*, although smaller effects have also been observed with genes 44, 46, and 47 (4). The present results demonstrate that at least three mutants of gene

TABLE 4. *Proflavine-induced ac^r mutant frequencies*

Expt	Net ac^r × 10^{4a}		ac^r ratio: *ts*/*ts*$^+$
	ts$^+$	*tsA80*	
1	10.7	7.0	0.7
2	10.2	8.1	0.8
3	6.3	9.6	1.5
Average	9.0	8.3	0.9

[a] Net *ac^r* equals the proflavine-induced less the control *ac^r* frequency. The control frequencies averaged 0.7 and 1.1% of the induced frequencies in *ts*$^+$ and *tsA80*, respectively.

30 (DNA ligase) produce only small effects upon frameshift mutation rates.

Our results conflict somewhat with a previous report (16) of a substantial increase in the proflavine-induced reversion rate of *rFC55* brought about by *tsB20*. We have no ready explanation for the differences observed, but we believe that the extensive nature of the tests reported here (and of many additional unreported tests) indicate that there is no evidence, as yet, for a substantial role for the DNA ligase gene in determining the spontaneous or induced frameshift mutation rate in bacteriophage T4. This conclusion takes into account careful attempts to avoid the confusion which might arise from the suppression of gene 30 mutations by *rII* mutations. In multiple cycle experiments, corrections could be made for this suppression by measuring selection coefficients. Furthermore, in single growth cycle experiments with proflavine, these selection artifacts are not likely to occur anyway, because the suppression of gene 30 defects by *rII* mutations which might be relieved by reversion of the *rII* mutation is no longer possible by the time the proflavine is added (13). Finally, the possible involvement of the host ligase in this system was sometimes avoided by the use of ligase-defective hosts, again with no effect observed on T4 frameshift mutation rates.

It is still possible that studies employing other gene 30 mutants, or testing the present mutants under different conditions, would reveal more substantial effects on frameshift mutation rates. Many of the experiments with our three *ts* mutants of gene 30, however, were performed at or very close to the nonpermissive temperature; we usually observed marked decreases in burst sizes with these *ts* mutants over the interval of 37 to 38 C, without observing corresponding increases in mutator effects.

The general theory of frameshift mutagenesis (17) proposes that the initial event consists of a strand interruption, which may or may not be followed by limited exonuclease degradation of one strand. Very localized melting and misannealing then occur, after which repair (carried out probably both by the DNA polymerase and ligase) freeze the misaligned condition into a mutational heterozygote. Certain *ts* mutations of the DNA polymerase gene have marked effects on the frameshift mutation rate, in agreement with this theory. Why, then, do not ligase defects also affect the frameshift mutation rate? The answer to this question is probably to be found in a special aspect of frameshift mutagenesis in bacteriophage T4, where the strand interruptions which are by far the most likely to initiate frameshift mutagenesis are the chromosome tips (14). In this system, therefore, ligase action need not be the final step in frameshift mutagenesis. However, ligase genes in organisms which do not employ circularly permuted chromosomes might be much more important determinants of the frameshift mutation rate than they are in T4.

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