JOURNAL OF VIROLOGY, Aug. 1976, p. 490-494
Vol. 19, No. 2
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Mutator Mutations in Bacteriophage T4 Gene *32* (DNA Unwinding Protein)

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Received for publication 25 March 1976

Bacteriophage T4 gene *32* encodes a DNA unwinding protein required for DNA replication, repair, and recombination. Gene *32* temperature-sensitive mutations enhance virtually all base pair substitution mutation rates.

Mutation rates are profoundly influenced by the functional state of proteins involved in DNA metabolism. In the case of bacteriophage T4 gene *43* (DNA polymerase), for example, temperature-sensitive alleles can increase spontaneous mutation rates by as much as three orders of magnitude (50) or decrease them by two orders of magnitude (18). Smaller changes have been observed with mutants of genes *30* (DNA ligase) (31), *32* (DNA unwinding protein) (8, 9, 17), *42* (dCMP hydroxymethylase) (13, 17), *44* (ATPase) (8), *46* and *47* (DNase) (8), *td* (thymidylate synthetase) (9, 49), *hm* (function unknown) (17, 23), and *px*, *y*, and *1206* (error-prone repair) (17, 23).

Descriptions of the specific mutational pathways whose rates are altered by mutations in a gene of DNA metabolism can be useful in understanding both the function of that gene and the mutational mechanisms affected. Specific mutational pathways can be assayed using well-characterized T4*rII* tester strains, and *rII* mutants capable of detecting mutation along specific base pair substitution pathways are continually being developed in this laboratory.

Bacteriophage T4 gene *32* encodes a DNA unwinding protein that is intimately involved in several aspects of DNA metabolism, including DNA replication, repair, and recombination (1-3, 7, 10, 22, 32, 35, 39, 41, 51, 53). Similar DNA unwinding proteins have been observed in other organisms, including the single-stranded DNA bacteriophages fd and M13 (4, 42), the double-stranded DNA bacteriophage T7 (44, 46), the bacterium *Escherichia coli* (47), the fungus *Ustilago maydis* (6), the plant *Lilium* (26), the spermatocytes of several mammals (including man) (27), cultured chicken embryo fibroblasts (30), and calf thymus (4, 47). Both experimental and theoretical bases therefore exist for assuming that such proteins act ubiquitously in DNA metabolism.

Since previous reports (8, 9, 17) already indicate that mutational lesions in gene *32* affect mutation rates, we have systematically surveyed the effects of such mutations upon the rates of virtually all base pair substitution pathways, and of frameshift mutation pathways as well. All base pair substitution pathways are affected. Possible physiological interactions between gene *32* and *rII* mutations (40) have been eliminated as causes of altered revertant frequencies of *rII* mutations in gene *32* *ts* backgrounds.

MATERIALS AND METHODS

Media. L broth and Drake agars (45) were used throughout.

Strains. All T4*rII* mutants are from the Urbana collection and reside in T4B backgrounds. Gene *32* *ts* mutants were obtained from Bruce Alberts; originally in T4D, they have been backcrossed four times into T4B. Double *rII-32ts* mutants were constructed by recombination and confirmed by backcrosses against each parent. *E. coli* B and BB are permissive hosts for *rII* mutants; B cells were used to detect the *r* plaque morphology phenotype, and BB cells (which, unlike B cells, do not permit the expression of the *rII* phenotype and do not select in favor of rII^+ revertants) were used to grow all stocks unless otherwise indicated. *E. coli* KB is a K-12 lambda lysogen that is nonpermissive for *rII* mutants and was used for selective plating of rII^+ revertants. *E. coli* CA265 is a K-12 lambda lysogen carrying a tyrosine-inserting amber suppressor. *E. coli* XA102c $su2^+$ is a glutamine-inserting amber suppressor that is nearly isogenic with the nonsuppressor XA90Nc su^- (24); these strains were obtained from Philip Farabaugh.

Reversion analysis. Stocks were initiated from fewer than 10^5 *rII* particles (at 10^4/ml) using BB cells at 10^7/ml and were grown on rotary shakers for 3.5 to 4 h at 32°C, or at 37°C where indicated. Lysis was completed with chloroform. At least four stocks were grown in parallel for each genotype to be tested, and all genotypes to be compared were also grown in parallel. The median rII^+ revertant frequency was computed; this method is often superior to taking the mean, since the latter procedure requires an arbitrary decision concerning the exclusion of "jackpot" stocks. Many experiments were repeated one or

more times, however, and in those cases the mean of the median revertant frequencies was computed. Under these conditions, differences in revertant frequencies of an *rII* mutant in different genetic backgrounds are reproducible within a two- to threefold factor.

To assay the conversion of an *rII* ochre codon to its homologous amber configuration ($OC \rightarrow AM$ = UAA → UAG = A:T → G:C), the ochre mutant was plated on CA265 cells. A sufficient number of plaques was then picked with sterile paper strips and spot-tested on both KB and CA265 cells to distinguish between revertants and convertants. Convertant frequencies were computed as above.

To avoid depressed plating efficiencies of rII^+ revertants because of excessive numbers of *rII* particles on a plate, the total number of particles was kept below 10^8/plate. The frequency of ts^+ revertants in gene *32 ts* stocks was always monitored by plating on BB cells at 43°C; the ts^+ frequency was almost always less than 10^{-4} and never greater than 10^{-3}. It was also helpful, to maximize the detection of rII^+ revertants in gene *32 ts* backgrounds, to use a supersoft top agar containing only 0.4% agar, together with a constant plating inoculum of about 10^8 log-phase lambda lysogen cells. All experiments also incorporated parallel efficiency-of-plating controls using standard suspensions of the appropriate genotypes; the efficiencies of plating of *rII* revertants or convertants were measured by plating a known number of particles on KB cells together with 10^8 particles of the corresponding parental *rII* mutant (or, when the rII^+ revertant frequency was greater than about 10^{-6}, together with about 10^8 particles of the nonreverting *rII* deletion *r1272*).

When selection controls were to be included, additional stocks were initiated with artificial mixtures of the *rII* mutant plus revertants of the same mutant added to a final frequency of about 10^{-1}; such mixtures were prepared both in uniformly *ts* and in uniformly ts^+ gene *30* backgrounds. The rII^+ frequencies were measured in both the initial inocula and the resulting stocks, coefficients of selection were calculated (31), and these were then used to correct the observed revertant frequencies.

RESULTS

The properties of the *rII* tester strains are shown in Table 1. The effects of gene *32 ts* mutations upon mutation rates are shown in Table 2. Virtually all base pair substitution mutation rates were increased in gene *32 ts* backgrounds, and on the whole all possible pathways (transitions and transversions at A:T and at G:C sites) were promoted to similar extents. However, two minor uncertainties persist. First, none of our *rII* tester strains assays G:C → A:T transitions exclusively. Second, although transversions were clearly induced at both G:C and A:T sites, ambiguities remain as to which specific transversions were induced (for instance, G:C → C:G or T:A).

Very small, and at best marginally significant, mutator and antimutator effects were observed with *rII* frameshift mutants. However, *tsG26* and *tsL67* were previously reported (8) to substantially increase the reversion rates of two other *rII* frameshift mutants, *rED144* and *r71*.

That these mutator activities are the direct consequence of the gene *32 ts* mutations, rather than of other unrecognized mutations introduced at the same time, is demonstrated by three criteria. First, the gene *32 ts* mutations were initially purified by four backcrosses against wild-type T4B. Second, ts^+ revertants of *rII-32ts* double mutants were previously shown to have lost mutator activity (8). Third, the rates of $rP7OC \rightarrow r^+$, $rP7AM \rightarrow r^+$ and $rP7OC \rightarrow AM$ decreased to within factors of less than twofold of their control (ts^+) values when backcrossed out of *tsG26* backgrounds.

Although physiological interactions may occur between *rII* and gene *32* mutations (40), general selection in favor of rII^+ revertants in a gene *32 ts* background can also be eliminated as a cause of the observed mutator effects by three criteria. First, significant selection was directly ruled out in the instances of *rUV20*, *rUV27*, and *rUV28*. Second, when the amber mutant *rUV183* was grown in the nearly isogenic nonlysogenic host strains XA90Nc su^- and XA102c $su2^+$ (inserting glutamine, which is "acceptable" at the *rUV183* codon), *tsG26* enhanced its reversion rate 11- and 24-fold, respectively. Third, when the plaque morphologies of *rII* mutants in gene *32 ts* backgrounds were examined on B cells, at 32°C in the case of *tsP7* and *tsG26* and at 37°C in the case of *tsL67* (which is substantially less temperature-sensitive than the other two mutants), the only combination showing a (slight) reduction in *r* plaque morphology was *rUV117-tsG26*, and more precise direct measurements of selection coefficients indicated the lack of significant selection in this example.

DISCUSSION

Our results, together with results reported previously (8, 9), clearly show that mutational defects in bacteriophage T4 gene *32* increase virtually all types of base pair substitution mutation rates. In addition, although we observed only very small effects upon frameshift mutation rates, large effects are also possible (8). These mutator effects are the specific results of gene *32* mutations and are not due to selection. Furthermore, they do not depend upon the absence of rII^+ function, since *tsG26* enhanced the reversion of the amber mutant *rUV183* in both suppressor and nonsuppressor hosts. Like gene *43* (DNA polymerase), therefore, gene *32* is a

TABLE 1. *Properties of rII tester mutants*

Mutant[a]	Reversion responses[b]						Permissible pathways of reversion or conversion[c]	Sources[d]
	BA	HA	PF	−T	−C	HT		
rUV183AM	+	−	−	+	±	−	A:T → G:C (A:T → Py:Pu)	15, 49
rUV191OC	+	−	−	+			A:T → G:C (A:T → Py:Pu)	15, 49
rUV199OC	+	−	−	+		−	A:T → G:C (A:T → Py:Pu)	15, 16, 49
rUV237	+	−	−	+			A:T → G:C (Pu:Py → Py:Pu)	15, 49
rUV247	+	−	−				A:T → G:C (Pu:Py → Py:Pu)	15
rP7OC → AM	+	−					A:T → G:C	20, 45
rUV7	+	+	−	−	+	+	G:C → A:T (G:C → Py:Pu)	15, 16, 49
rUV13	+	+	−	−	+	+	G:C → A:T (G:C → Py:Pu)	15, 16, 49
rUV48	+	+	−	−	+	+	G:C → A:T (G:C → Py:Pu)	15, 16, 49
rUV279	+	+	−			+	G:C → A:T (Pu:Py → Py:Pu)	15, 49
rSM94	+	+	−	−	+	+	G:C → A:T (G:C → Py:Pu)	21, 49
rUV117	−	−	±	+		−	A:T → Py:Pu	15, 16, 45, 49
rP7OC → r$^+$	−	−	−	+		−	A:T → Py:Pu	20, 45
rUV74	−	−	−	±	−	+	G:C → Py:Pu	15, 45, 49
rP7AM → r$^+$	−			±		+	G:C → Py:Pu (A:T → Py:Pu)	20, 45
rUV2	−	−	+			−	Frameshift mutation	15, 16
rUV20	−	−	+			−	Frameshift mutation	15, 16
rUV27	−	−	+		±	−	Frameshift mutation	15, 16
rUV28	−	−	+	−	±	−	Frameshift mutation	15, 49

[a] Where not indicated, reversion to r^+ was assayed. *OC → AM* indicates the conversion of an ochre codon to the homologous amber codon.

[b] BA, Base analogues (2-aminopurine and/or 5-bromouracil); HA, hydroxylamine; PF, proflavin; −T, thymineless mutagenesis; −C, cytosineless mutagenesis induced by *ts* mutations in gene *42* (W. E. Williams and J. W. Drake, unpublished data); HT, heat (R. H. Baltz, P. M. Bingham, L. S. Ripley, unpublished data); +, reversion or conversion induced; −, reversion or conversion not induced; ±, weak or equivocal reversion or conversion.

[c] In general, only frameshift mutants are revertible by proflavin. A mutant revertible by base analogues but not by hydroxylamine can revert by the transition A:T → G:C, but not by the reverse transition; it may also be able to revert by transversions. A mutant revertible by hydroxylamine can revert by G:C → A:T transitions, and may in addition be able to revert by the reverse transition and by transversions. Thymineless mutagenesis induces both transitions and transversions, but only at A:T base pairs (49). Cytosineless mutagenesis induces only G:C → A:T transitions strongly, although it induces the reversion of frameshift mutants weakly (W. E. Williams and J. W. Drake, unpublished data). Heat induces both transitions and transversions (perhaps only G:C → C:G) at G:C base pairs (16, 21; and unpublished data of R. H. Baltz, P. M. Bingham, and L. S. Ripley). *rUV183* is an amber mutant unable to revert by transversion at the G:C base pair of the amber codon because of the unacceptability of tyrosine at that site. Pathways outside of parentheses are definitely permissible, whereas those within parentheses may or may not be permissible.

[d] Plus numerous unpublished data of R. H. Baltz, P. M. Bingham, L. S. Ripley, and W. E. Williams.

major determinant of the fidelity of DNA synthesis in bacteriophage T4. It remains to be determined whether rates of chemically induced mutation are also influenced by the functional state of gene *32*, as they are by that of gene *43* (18, 19), and whether DNA unwinding proteins are also involved in misrepair mutagenesis (23).

The T4 DNA unwinding protein is autogenously regulated (33) and is required in stoichiometric amounts for T4 replication (48). It is readily cleaved in vitro to produce a large fragment that lowers DNA melting temperatures more markedly than does the native protein (5, 25, 36), but an in vivo role of such cleavage remains hypothetical. It is not yet clear which of the several interactions between DNA unwinding proteins and other cellular constituents are responsible for maintaining the accuracy of DNA synthesis. The T4, T7, and *E. coli* DNA unwinding proteins are required, often absolutely, for the replication of both single-stranded and double-stranded DNA (2, 14, 29, 41). They self-associate, and many copies bind cooperatively to single-stranded DNA and presumably maintain it in an extended configuration suitable for template (and perhaps also primer) activity (2, 3, 11, 12, 52). The interaction between DNA and the unwinding proteins of phages fd and T4 (but not of *E. coli*) may be mediated by stacking between bases and tyrosyl residues (5, 43). Highly specific interactions also occur between DNA unwinding proteins and DNA polymerases (29, 37, 38, 44, 47). Indirect evidence suggests a further interaction between the T4 DNA unwinding protein and the T4 DNA ligase (40) and perhaps with other as yet unidentified T4 proteins (34).

TABLE 2. *Effects of gene 32 ts mutations on reversion rates of rII tester mutants*

Tester mutant[a]	Probable mutational pathway assayed[a]	Spontaneous frequency per 10^8 particles	Factor of increase with indicated gene *32* allele[b]		
			tsP7	*tsL67*	*tsG26*
rUV183	A:T → G:C	80	8.0 (3)	11	15
rUV191	A:T → G:C	3.4	6.6	3.7	12
rUV199	A:T → G:C	69	8.2 (2)	4.5	5.1
rUV237	A:T → G:C	2.7	5.4 (2)	4.1	9.8
rUV247	A:T → G:C	32	4.2	2.4	2.1
rP7OC → AM	A:T → G:C	0.60	14	4.7	7.0
rUV7	G:C → A:T	36	19	3.2	7.8
rUV13	G:C → A:T	4.5	2.3 (2)	2.1	2.8
rUV48	G:C → A:T	30	5.4 (2)	2.3	6.1
rUV279	G:C → A:T	74	6.5	2.4	9.7
rSM94	G:C → A:T	42	0.8		
rUV117	A:T → Py:Pu	1,470	1.4 (2)	2.4	5.5 (2)[c]
rP7OC → r^+	A:T → Py:Pu	0.70	9.3 (2)	1.1	6.1 (3)
rUV74	G:C → Py:Pu	25	3.3 (3)	8.5[d]	2.5 (2)
rP7AM → r^+	Transversions	1.4	7.2 (3)	1.3	8.2 (3)
rUV2	Frameshifts	80	1.6		
rUV20	Frameshifts	10.9	1.1	1.5[c, d]	1.2[c]
rUV27	Frameshifts	2,200	2.0		0.58[c]
rUV28	Frameshifts	47	0.66 (2)[c]	1.7[c, d]	1.5[c]

[a] See Table 1. Other pathways are possible with some of these mutants.
[b] Numbers in parentheses indicate numbers of times the measurement was repeated (see Materials and Methods). Only values greater than about 2.0 or less than about 0.5 are highly significant.
[c] Corrected for selection. The correction factor was never greater than 25%.
[d] Measurements performed at 37°C (all others performed at 32°C).

There are two obvious ways in which the T4 DNA unwinding protein could promote accurate DNA synthesis: by assuring an alignment of the DNA polymerase and the template strand (and perhaps also the primer strand) which is optimal for discrimination against errors in base pairing, and by assuring that the DNA polymerase is in the optimal tertiary configuration. Both may be important, and both may depend critically upon the precise nature of the polymerase/unwinding protein interaction. (It may be especially significant that DNA unwinding proteins markedly and specifically affect the 3′-exonuclease proofreading functions of both the T4 and the *E. coli* DNA polymerases [28, 38].) It will therefore be of considerable interest to obtain mutants, of either the polymerase or the unwinding protein, which are specifically perturbed in this interaction.

ACKNOWLEDGMENTS

This work was supported by grant VC-5 from the American Cancer Society, grant BMS-71-01243 from the National Science Foundation, and grant 04886 from the National Institute of Allergy and Infectious Diseases.

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