Reversion of Frameshift Mutations Stimulated by Lesions in Early Function Genes of Bacteriophage T4

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Temperature-sensitive (*ts*) mutants representative of a number of genes of phage T4 were crossed with *rII* mutants to allow isolation of *ts*, *rII* double-mutant recombinants. The *rII* mutations used were characterized as frameshift mutations primarily on the basis of their revertability by proflavine. For each *ts*, *rII* double mutant, the effect of the *ts* mutation on spontaneous reversion of the *rII* mutation was determined over a range of incubation temperatures. A strong enhancement in reversion of two different *rII* mutants was detected when they were combined with *tsL56*, a mutation in gene 43 [deoxyribonucleic acid (DNA) polymerase]. Three other mutants defective in gene 43 enhanced reversion about fourfold. Two mutations in gene 32, which specifies a protein necessary for DNA replication, enhanced reversion about 5-fold and 18-fold, respectively. Two additional mutations in gene 43 and two in gene 32 had no effect. Fivefold and threefold enhancements in reversion were also found with mutations in genes 44 (DNA synthesis) and 47 (deoxyribonuclease), respectively. No significant effect was found with mutations in seven additional genes. The results of other workers suggest that frameshift mutations arise from errors in strand alignment during repair synthesis occurring at chromosome tips. Our results show that such errors can be enhanced by mutations in the DNA polymerase, the gene 32 protein, and the enzymes specified by genes 44 and 47. This implies that these proteins are employed in the repair process occurring at chromosome tips and that mutational errors in these proteins can lead to loss of ability to recognize and reject strand misalignments.

Streisinger et al. (20) have shown in phage T4 that frameshift mutations often occur in regions of repeated base sequence. On the basis of this observation, they suggested that misalignment between the two complementary strands of deoxyribonucleic acid (DNA) during repair of single-strand gaps may tend to produce frameshift mutation. Lindstrom and Drake (16) demonstrated that in phage-infected cells frameshift mutations arise in the terminally redundant tips of progeny chromosomes. They also suggested that these mutations are caused by errors of alignment occurring in terminal repair processes.

The present study was undertaken to determine which phage-specified proteins are involved in the repair processes that generate frameshift mutations. Twenty-two temperature-sensitive (*ts*) mutants defective in 11 different genes (Table 1) were tested for effects on the reversion of particular *rII* mutations which were characterized as frameshift mutations. Crick et al. (7) have shown in phage T4 that reversion of an initial frameshift

mutation occurs as the result of a second frameshift mutation of opposite sign to the initial one.

MATERIALS AND METHODS

Phage strains. All of the phage mutants used in this study were derived from T4D, except for *r375* which comes from T4B. The *ts* mutants were isolated by R. S. Edgar, and some of the initial work with them has been presented by Edgar and Lielausis (12). The isolation and characterization of *rED144* and *r71* were first reported by Edgar et al. (11). Double mutants were isolated from *ts* × *r* crosses performed in *Escherichia coli* B/5. They were distinguished by their *r* plaque morphology on *E. coli* S/6 at 25 C, their inability to form plaques on *E. coli* S/6 at 42 C, and their inability to grow on *E. coli* K λ at 25 C.

Revision studies. A modified M-9 medium was used for liquid culture in all experiments. This contained the following ingredients, per liter of distilled water: 1.0 g of NH₄Cl, 0.25 g of MgSO₄, 3.0 g of KH₂PO₄, 5.8 g of Na₂HPO₄, 0.5 g of NaCl, 2.7 mg of FeCl₃·6H₂O, 20 mg of L-tryptophan and 4.0 g of glucose. The MgSO₄, FeCl₃, glucose, and tryptophan were each autoclaved separately and then added to the remaining salts. A 10-ml amount of this medium was

inoculated with bacteria from a fresh overnight culture to give a concentration of about 10^6 bacteria per ml. After about 1 hr, 200 to 500 phage were added from a clonally derived phage suspension. The culture was incubated with bubbling in a water bath adjusted to a specific temperature. After 8 to 12 hr, during which clearing had generally occurred, a few drops of chloroform were added. The lysate was then plated out at appropriate dilution on *E. coli* S/6 at 25°C to measure the total progeny titer and on *E. coli* K λ at 25°C to measure the titer of revertants. Generally 12 cultures were grown at one time at a given temperature. Each set included, in addition to the *ts,r* double-mutant cultures, appropriate single-mutant *r* cultures for comparison. The final titers of *rED144*, *r71*, and *r375* grown in this manner averaged 2.5×10^{10} , 2.5×10^{10} , and 2.0×10^{10} , respectively. The titers attained in the cultures inoculated with double-mutant phage varied with temperature. Reversion indexes of *r* mutants and *ts,r* double mutants were determined by taking the ratio of the *r*⁺ or *ts,r*⁺ titer (determined on *E. coli* K λ at 25°C) to the total phage titer (determined on *E. coli* S/6 at 25°C).

When plating for revertants on *E. coli* K λ , care was taken to place no more than 2.5×10^8 total phage on a plate. With numbers larger than this, the indicator bacteria were killed to an extent that interfered with the reliability of revertant counts. In each experiment, a correction was made for the small differences in efficiency of plating of the *r*⁺ or *ts,r*⁺ revertants on *E. coli* K λ as compared to *E. coli* S/6.

Chemical mutagenesis. For proflavine mutagenesis, the procedure of Barnett et al. (3) was followed. In treating with 2-aminopurine (2AP) and 5-bromodeoxyuridine (5-BdU), *r* mutant stocks were grown as described above except that the mutagens were added at the time of addition of phage to the culture to give final concentrations of 1 mg of 2AP per ml or 0.1 mg of 5-BdU per ml.

Temperature-insensitive revertants (*ts*⁺*r*) of *ts, r* double mutants. To obtain temperature-insensitive revertants, *ts, r* double mutants were plated at high titer on *E. coli* S/6 at 42°C. Plaques occurring under these conditions were picked, resuspended, and further tested for growth at 42°C and for retention of the *rII* character. After testing, stocks were prepared from single isolated plaques.

RESULTS

Reversion of *rED144*. Edgar et al. (11) isolated *rED144* as a spontaneous mutant and mapped it in the *rIIA* cistron. The spontaneous reversion index of this mutant was measured at different temperatures over the range of 27 to 40°C (Fig. 1A) and gave an average value of 5.40×10^{-8} . In the presence of 2AP (1 mg/ml) or 5-BdU (0.1 mg/ml), no increase in reversion was observed (Table 1). Proflavine, however, did increase reversion of *rED144* more than 20-fold over untreated cultures (Fig. 2). *rED144* was also observed to be nonleaky. This pattern of properties has been

shown to be characteristic of frameshift mutations by Orgel and Brenner (17) and Crick et al. (7).

Twenty-two different *ts* mutants defective in 11 different genes were crossed with *rED144*, and double mutants were isolated (Table 1). The reversion index of *rED144* in each of these double mutants was measured, in most cases at two or more different temperatures. The increases in reversion for all of the *ts,rED144* double mutants tested are recorded in Table 1.

Enhancement of *rED144* reversion by gene 32 *ts* alleles. Gene 32 specifies a protein which binds cooperatively to single-stranded DNA and is necessary for replication and recombination (1). Two of the four mutations in gene 32, *tsG26* (Fig. 1A) and *tsL67* (Fig. 1B), caused average increases in reversion of 17.5-fold and 4.9-fold, respectively (Table 1). The remaining two mutants, *tsP40* and *tsP7* had no significant effect.

Two "*ts*⁺" revertants of *tsG26, rED144* able to grow at high temperature were selected. These are referred to as *tsG26R1, rED144* and *tsG26R2, rED144*. Stocks of these were tested for reversion of *rED144*. As shown in Fig. 1A, the reversion index in *tsG26R1, rED144* had returned to the level characteristic of *rED144* before addition of the *tsG26* marker, whereas with *tsG26R2, rED144* the level was 3.4-fold higher than with *rED144*. If reversion of the *ts* lesion can restore the normal *r* reversion index, as it does in the case of *tsG26R1, rED144*, this implies that the increase in *r* reversion in the double mutant was due specifically to the *ts* lesion and not to other unidentified mutations. The intermediate results with *tsG26R2, rED144* suggest that reversion of the *ts* phenotype may occur by mutation to another missense codon at the same site or by suppression.

Enhancement of *rED144* reversion by gene 43 *ts* alleles. Gene 43 codes for DNA polymerase (8, 21). Six different gene 43 *ts* mutations were tested for effects on the reversion of *rED144* (Table 1). *tsL56* increased reversion 32-fold. The data for this allele are shown in Fig. 1C. Three isolates of *tsL56, rED144* selected for ability to form plaques at high temperature gave *rED144* reversion indexes about twofold higher than the standard index for *rED144*. This implies that most of the increase in *r* reversion in the double mutant was due to the *ts* lesion, but reversion of the *ts* mutation is not usually back to true wild type.

Three other gene 43 *ts* mutations (*tsL91, tsCB87* and *tsL88*) enhanced reversion of *rED144* three- to fourfold (Table 1). These results are plotted in Fig. 1D. A temperature-insensitive revertant of *tsL91, rED144* gave an *rED144* reversion index similar to the control level. The

FIG. 1. Reversion of *rED144* in the presence of different *ts* mutations, revertants of these mutations, and in the wild-type (*ts*⁺) background, measured over the temperature range of 27 to 40°C. The points indicated by an asterisk represent the average of two determinations. All other points represent single determinations.

remaining two mutations in gene 43 (*tsCB123* and *tsG37*) had no effect on *rED144* reversion.

Enhancement of *rED144* reversion by *ts* alleles of genes 44, 46, and 47. Gene 44 is required for DNA synthesis, (13) but its function is otherwise unknown. Genes 46 and 47 have been characterized as specifying deoxyribonucleases (22). The one allele tested in gene 44 (*tsB110*) increased reversion of *rED144* fivefold (Table 1). Of two alleles tested in gene 47, one (*tsL86*) caused a threefold increase in *rED144* reversion, and the other, *tsB10*, had no effect (Table 1). When "*ts*⁺" revertants of *tsB110*, *rED144* and *tsL86*, *rED144* were tested, reversion of *rED144* returned to the standard level. Of two mutations tested in gene 46, one (*tsL109*) had no effect, and the other (*tsL166*) was associated with an 11-fold increase (Table 1). However "*ts*⁺" revertant iso-

lates of *tsL166*, *rED144* also showed a high level of *rED144* reversion. There are two possible explanations for this behavior. First, *tsL166* may not be causing the high reversion rate in the double mutant, and the effect is due to an unidentified extraneous marker introduced when the double mutant *tsL166*, *rED144* was formed. Secondly, *tsL166* may be responsible for the increase, but the two temperature-insensitive revertants tested were not true back mutations and retained the mutagenic property.

Other genes. The *ts* mutations tested in gene 30 (polynucleotide ligase), 36 (tail fiber structure), 39 (DNA synthesis), 45 (DNA synthesis), 49 (head structure) and 56 deoxycytidine triphosphatase had no significant effect on the reversion of *rED144*.

Reversion of *r71*. Mutant *r71* was isolated as a

FIG. 2. Increase in reversion of *r71* (○) and *rED144* (●) in the presence of various concentrations of proflavine. Each point was calculated by taking the ratio of the reversion index of the *r* mutant in the presence of proflavine to the reversion index of the mutant in the absence of proflavine measured in the same experiment. The data given here are from four experiments.

spontaneous mutant (11). It is located in the *rIIA* cistron about one map unit from *rED144*. The spontaneous reversion index of the mutant was measured at the different temperatures indicated in Fig. 2, giving an average value of 1.07×10^{-8} . Reversion was stimulated an average of about 40-fold by concentrations of proflavine from 6 to 16 $\mu\text{g/ml}$ (Fig. 3). This stimulation is in the range expected for frameshift mutations (17). No increase was observed when the mutant was grown in 0.1 mg of 5-BdU per ml. In the studies of Champe and Benzer (6), this treatment increased guanine plus cytosine (GC) $\rightarrow$ adenine plus thymine (AT) transitions 100- to 10,000-fold and increased AT $\rightarrow$ GC transitions about 10-fold or less. Treatment with 2-AP was at 1 mg/ml. In the Champe and Benzer studies (6), this concentra-

tion increased both kinds of transition 100- to 10,000-fold, and we have found that reversion of *r375* (a mutant shown by these authors to revert by AT $\rightarrow$ GC transition) was stimulated 7,320-fold under our conditions. Reversion of *r71* was enhanced a small amount (5.7-fold) with this treatment. The observation that *r71* did not show greater than 100-fold enhancement with 2-AP (and no enhancement with 5-BdU) suggests that it is not a transition mutation. The 40-fold average increase with proflavine taken together with the observation that *r71* is nonleaky suggests that this mutant is of the frameshift type. The small effect of 2-AP remains unexplained.

Eight different *ts* mutations in six different genes were combined with *r71* to form double mutants, and the effect of the *ts* allele on reversion of *r71* was measured. Each of four *ts* mutations in genes 30, 45, 46, and 56 had no significant effect (Table 1). Of the two *ts* mutations in gene 32, one (*tsL67*) increased reversion an average of 6.3-fold (Table 1) and the other (*tsG26*) caused only a small, 2.5-fold increase. Two mutations in gene 43 were tested. One of them (*tsL56*) caused on the average a 6,230-fold increase (Fig. 3). The other (*tsCB87*) caused a 4.7-fold increase. A "*ts*+" revertant of *tsL56,r71* was tested for reversion of *r71*. As shown in Fig. 3, the reversion index of this strain is 32-fold higher than the *r71* control but 194-fold less than with *tsL56,r71*. This result is similar to that obtained with *ts*⁺ revertants of *tsL56,rED144* and again implies that revertants of the *tsL56* allele are often not true wild type.

Reversion of *r375*. This mutant has been characterized as reverting by AT $\rightarrow$ GC transition (6). The strong response to 2-AP treatment (7,320-fold increase in reversion) and the weak response (7.7-fold increase) with 5-Bd observed in our laboratory (Table 1) are in agreement with this characterization. Double mutants were isolated consisting of *r375* in combination with *tsG26* (gene 32), *tsL13* (gene 42), *tsL109* (gene 46), and *tsL86* (gene 47). These were used in a preliminary screening for stimulation of transition mutation by alleles of genes other than gene 43 (DNA polymerase). Mutagenic effects of gene 43 alleles on transitions have already been demonstrated (19). However, no significant increases in reversion were observed in these double mutants.

Crosses. The effects of *ts* alleles on frameshift mutation and recombination will be compared below. Most of the data on recombination have been presented previously (4, 5). However, results not presented before with two *ts* mutations should be mentioned here: *tsL56* and *tsL67*, which showed significant mutagenic activity, were also tested for effects on recombination. The following crosses were carried out: *tsL56,rED144* $\times$

TABLE 1. Stimulation of reversion of *rED144*, *r71*, and *r375* by temperature-sensitive mutations in different genes

Gene	Mutant	<i>rED144</i>		<i>r71</i>		<i>r375</i>	
		No. of tests	Increase ^a	No. of tests	Increase ^a	No. of tests	Increase ^a
30	<i>tsB20</i>	2	1.0	2	2.2		
	<i>tsA80</i>	2	1.6	2	1.0		
32	<i>tsG26</i>	7	17.5	1	2.5	2	2.4
	<i>tsG26R1</i>	4	1.0				
	<i>tsG26R2</i>	1	3.4				
	<i>tsL67</i>	9	4.9	6	6.3		
	<i>tsL67R1</i>	2	1.2				
	<i>tsL67R2</i>	2	0.9				
	<i>tsL67R3</i>	2	1.2				
	<i>tsP40</i>	3	1.5				
	<i>tsP7</i>	2	1.3				
36	<i>tsN41</i>	3	1.7				
39	<i>tsG41</i>	1	0.5				
42	<i>tsL13</i>					1	0.5
43	<i>tsL56</i>	9	32	8	6,230		
	<i>tsL56R1</i>	4	1.8	2	32		
	<i>tsL56R2</i>	4	2.1				
	<i>tsL56R3</i>	4	2.0				
	<i>tsL91</i>	4	4.2				
	<i>tsL91R1</i>	2	1.5				
	<i>tsCB87</i>	4	3.1	2	4.7		
	<i>tsL88</i>	2	3.9				
	<i>tsCB123</i>	2	1.5				
	<i>tsG37</i>	2	1.1				
44	<i>tsB110</i>	2	4.9				
	<i>tsB110R1</i>	2	1.3				
45	<i>tsL159</i>	2	0.7				
46	<i>tsL109</i>	2	0.9	1	0.5	7	0.6
	<i>tsL166</i>	9	10.8				
	<i>tsL166R1</i>	2	8.2				
	<i>tsL166R2</i>	1	5.7				
47	<i>tsL86</i>	4	3.0			1	0.3
	<i>tsL86R1</i>	2	0.9				
	<i>tsB10</i>	1	1.0				
49	<i>tsC9</i>	2	1.3				
56	<i>tsA90</i>	2	1.2	1	0.6		
Wild-type	+2AP	3	1.0	4	5.7	1	7,320
Wild-type	+5-BdU	3	1.2	3	0.5	2	7.7
Wild-type		26		10		13	

^a This is calculated by taking the ratio of the average reversion index of the *r* mutant in the presence of the *ts* allele to the average reversion index of the *r* mutant in the *ts*⁺ background. The *ts*⁺*r* → *ts*⁺*r*⁺ values were 5.40×10^{-8} for *rED144* based on 26 measurements, 1.07×10^{-8} for *r71* based on 10 measurements, and 7.95×10^{-8} for *r375* based on 13 measurements. Revertants of *ts* mutants are indicated by an *R* following the mutant designation.

tsL56, *r71*, *tsL67*, *rED144* × *tsL67*, *r71*, and, as a control, *rED144* × *r71*. The recombination percentages obtained for each cross were 3.59, 0.583, and 1.03, indicating the *tsL56* (gene 43) enhances recombination and *tsL67* (gene 32) reduces recombination.

DISCUSSION

Gene 43. Six different *ts* mutants defective in gene 43 were tested. One mutant (*tsL56*) had a

strong mutagenic effect. Three others (*tsL91*, *tsCB87*, and *tsL88*) were mildly mutagenic, and two (*tsCB123* and *tsG37*) showed no effect. *tsL56* and *tsL88* were shown by Speyer et al. (19) to revert strongly certain *rII* mutants of the amber type. By studying the conversion of ochre to amber mutations, they showed that both *tsL88* and *tsL56* can enhance AT → GC transition 20- to 800-fold. Furthermore, *tsL56* was shown both by Speyer et al. (19) and Drake et al. (10) to be

FIG. 3. Reversion of *r71* in the presence of *tsL56* (gene 43), a revertant of *tsL56* (labeled *tsL56RI*), and in a wild-type background (labeled *ts+*). The points indicated by an asterisk represent the average of two determinations.

strongly mutagenic for the formation of *r* mutants in a wild-type strain. Drake reported that *tsL56* increased the spontaneous level of *r* mutants from 4×10^{-4} to 120×10^{-4} . *tsL88* only enhanced the level to 8×10^{-4} . In addition, Speyer et al. (19) reported that, of the *rII* mutants induced in a wild-type strain, about half were not revertable by 2-AP, indicating that they had not arisen by transition mutation. Greater than 90% of the *r* mutants showed enhanced reversion in the presence of the mutator that produced them. These results suggest that, in addition to transitions, *tsL56* and *tsL88* also promote other changes such as transversions and frameshifts. This conclusion is consistent with our finding that *tsL56* and *tsL88* do in fact enhance frameshift mutation.

Two *rII* frameshift mutations (*rFC7* and *rFC42*) were tested by Speyer et al. (19) for stimulation of reversion by *tsL56* and *tsL88*, but no enhancement was found. We found significant enhancements by using the same polymerase alleles but with different *rII* frameshift mutations (32- and 6,230-fold with *tsL56* and 3.9-fold with *tsL88*). This suggests that reversion of some, but not all, frameshift mutations can be enhanced by particular mutator alleles and also that the levels of enhancement can vary. Similar site

specificity was also observed for mutator effects on base substitution mutation.

Drake et al. (10) also list effects of a number of additional *ts* mutations in gene 43 on reversion of *UV6*, an *rII* frameshift mutation, and *UV117*, a probable frameshift mutation. Nine *ts* mutations caused no significant increase with *UV6*, but one (*tsL98*) increased reversion 92-fold. Of nine *ts* mutations tested against *UV117*, two (*tsL106* and *tsL97*) increased reversion 3.8- and 4.4-fold, respectively, whereas the other *ts* mutations had little or no effect.

The observations of these other workers together with our own indicate that mutational alteration of the DNA polymerase can enhance both base substitution and frameshift mutation and that the mutagenicity of individual polymerase alleles and the mutability of individual DNA sites vary widely.

On the mechanism of frameshift mutation. Freese and Freese (14) studied the combined effect of a mutagenic polymerase and various mutagenic chemical agents on base substitution mutation. On the basis of their results, they suggested that the DNA polymerase may efficiently recognize and reject mispairs occurring during replication between the template strand and the incoming nucleotide and that a mutated polymerase might be less able to do this.

The results presented in this paper can be explained if the postulated ability of the polymerase to recognize and reject mispaired bases is extended also to strand misalignments. The observation of Streisinger et al. (20) that frameshift mutations tend to occur in regions of repeating DNA sequence indicates that the initiating event in frameshift mutation is the formation of a misalignment between the two strands of DNA near a nick or a gap in one strand. Support for this hypothesis comes from the observation of Lamfrom and Sarabai (15) that a polynucleotide ligase mutant (*tsB20*) increased the level of frameshift mutation by about 14-fold, when growth was carried out in the presence of proflavine (in contrast to our experiments with *tsB20* where no proflavine was used and no increase in reversion was seen). They postulated that when a mutant ligase is present in a cell single-strand breaks would be repaired inefficiently, thus enhancing the possibility of misalignments.

Lindstrom and Drake (16) recently presented evidence that frameshift mutations occur at the tips of phage T4 chromosomes and, taking into account the evidence of Streisinger et al. (20), speculated that these mutations arise from misalignment of the two DNA strands during repair synthesis.

Our experiments suggest that this repair synthesis employs both the DNA polymerase speci-

fied by gene 43 and the gene 32 protein, as well as the products of genes 44 and 47. Gene 43 lesions could enhance frameshift mutation by diminishing the ability of the polymerase to recognize and reject strand misalignments during repair synthesis. In the case of a normally functioning polymerase, recognition of a misalignment might cause a pause in DNA synthesis, and, since the misaligned state and the correctly aligned state are probably in equilibrium, the pause could be followed by renewed synthesis after correct alignment has occurred.

Defects in the proteins specified by genes 32, 44, and 47 could also diminish the efficiency with which misalignments are recognized and rejected, if these proteins play a role in the repair of chromosome tips. Baldy (2) has shown that genes 32, 43, and 47 are involved in the repair of UV-induced lesions which suggests that they might also be employed in other repair processes. Genes with radiation-sensitive alleles also showing mutator effects have been described in other organisms [see Drake (9) for review].

The observation that different mutations within genes 32, 43, and 47 have different mutagenic effects on *rEDI44* suggests that the ability to reject misalignments may be related to the specific nature of the protein lesion. That *r* mutations at different sites are stimulated to revert to different extents by *tsL56* suggests that neighborhood sequence may govern the stability of misalignments and thereby affect the efficiency of rejection by the mutant polymerase. For example, mutant *r71* may be located in a region allowing stable misalignments to be formed. These may not be easily rejected by the mutant polymerase but may be efficiently rejected by the normal enzyme.

Effects of *ts* mutations on recombination compared to mutagenicity. Three mutants used in the present study were defective in recombination. Over the range of temperatures employed here, *tsL109* (gene 46) and *tsL86* (gene 47) have been shown to decrease recombination to about half the normal value (5). As reported above, *tsL67* (gene 32) also decreases recombination to a similar extent. *tsL67* (gene 32) and *tsL86* (gene 47) stimulated reversion of *rEDI44* fivefold and threefold, respectively, whereas *tsL109* was non-stimulatory. These results suggest that the products of genes 32 and 47 catalyze reactions common to both recombination and the repair of chromosome tips.

Alleles with increased mutability and decreased ability to recombine have also been reported in *Bacillus subtilis* by Prozorov and Barabanshchikov (18).

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