SUPPRESSION OF T4D LIGASE MUTATIONS BY *rIIA* AND *rIIB* MUTATIONS*

BY HILLARD BERGER† AND ANDRZEJ W. KOZINSKI‡

JOHNS HOPKINS UNIVERSITY, BALTIMORE, MARYLAND, AND UNIVERSITY OF PENNSYLVANIA, PHILADELPHIA

Communicated by Charles Yanofsky, September 11, 1969

Abstract.—The introduction of *rII* mutations into polynucleotide ligase *amber* mutants (gene 30) of T4D bacteriophage results in the restoration of phage growth in the nonpermissive host *E. coli* B. When cells are mixedly infected with *rII*⁺ -ligase[−] and *rII*-ligase[−] phage, growth is reduced, indicating that the *rII* mutations are recessive to the *rII*⁺ alleles. Infection with the *rII*-ligase[−] double mutants results in nearly complete restoration of phage DNA synthesis and prevents the extensive degradation of parental phage DNA observed after infection with ligase[−] single mutants. Similar results are observed when cells are infected with ligase[−] mutants and chloramphenicol is added five minutes post infection.

Introduction.—Gene 30 of T4 bacteriophage is responsible for the synthesis of a phage-induced polynucleotide ligase which catalyzes, *in vitro*, the repair of phosphodiester bond scissions in bihelical DNA.¹ Phage containing *amber* mutations in gene 30 produce little or no progeny in the nonpermissive hosts *E. coli* B or *E. coli* B23 and exhibit markedly reduced DNA synthesis.^{1, 2} Kozinski² has shown that the addition of chloramphenicol approximately five minutes after infection of nonpermissive cells with a gene 30 *amber* mutant restores nearly normal levels of DNA synthesis. When nonpermissive cells are infected with ligase[−] phage, there is extensive endolytic degradation of the phage DNA. This extensive degradation is not observed when chloramphenicol is added five minutes after infection or in cells infected with wild-type phage.² In addition, the phage DNA produced in the presence of chloramphenicol is of large size. When chloramphenicol is subsequently removed in order to allow maturation, phage yields of approximately 25 per cent of the wild type are produced.^{3, 4} These observations suggest that a phage-coded endonuclease is responsible for DNA degradation (nicking) and that the nicks are normally repaired by the phage ligase. Inhibition by chloramphenicol of the synthesis of nicking enzyme(s) reduces the requirement for ligase function and restores phage viability. This interpretation leads to the prediction that phage “suppressor” mutations can be found which permit growth of ligase[−] phage and are not within the ligase structural gene. Presumably these suppressor mutations would decrease the level of phage-directed endonuclease activity.

In this communication we report the results of *in vivo* and *in vitro* experiments which show that *rII* mutations act as suppressors of ligase[−] mutants of phage T4.

Materials and Methods.—**Bacterial strains:** *E. coli* B was used as the nonpermissive host for the *in vivo* experiments. *E. coli* B23⁵ was used for the *in vitro* studies. *E. coli* CR63 was used to prepare stocks and as the permissive host for all phage containing *amber* mutations. The *rII* reversion studies were done by plating *rII*-ligase[−] phage on

E. coli K-12 (λ h) or *E. coli* CR63 (λ h). *E. coli* S/6 (nonpermissive for *amber* mutants) was used as host for spot testing of *ligase*⁻ and *rII*-*ligase*⁻ mutants.

Phage stocks: T4D was used throughout. The mutants used were *amX39* (gene 30),⁶ *amE605* (gene 30), *r2-19* (*rIIA*), *r70* (*rIIA*), *r59* (*rIIA*), *r2-20* (*rIIA*), *rb50* (*rIIB*), and *r48* (*rI*). All of the *rII* mutants are revertable missense mutants.^{7, 8} Multiple mutation stocks were prepared by recombination. The double mutants were identified by their inability to grow in *E. coli* CR63 (λ h) and low plating efficiency on *E. coli* B (see *Results* and ref. 11).

Physico-chemical characterization of phage and phage DNA: The procedures for isotopic labeling, net DNA synthesis measurements, isolation of DNA, and analysis in CsCl and sucrose gradients have been previously described.^{2, 9} Where appropriate, details are given in the figure legends.

Results.—Growth of *rII*-*ligase*⁻ double mutants in *E. coli* B: The results of burst size and "transmission coefficient"¹⁰ measurements with two different gene 30 *amber* mutants and with a group of double mutants containing a gene 30 *amber* mutation and a *rII* mutation are shown in Table 1.

At high multiplicities of infection,¹¹ all of the *rII*-*ligase*⁻ double mutants examined have burst sizes of approximately 30–40 per cent of the cells infected with an *rII* mutant alone. In addition, all of the infected bacteria yield phage (transmission coefficient = 1). In contrast, less than 1 per cent of the cells infected with the *ligase*⁻ single mutants *amX39* and *amE605* produce phage. In these cases the burst size is approximately 0.01 per cent of the control. One experiment was performed using a *ligase*⁻ *r48* (*rI*) double mutant. No suppressor activity was observed with this combination of mutations. If T4 induced *ligase* compensates for a nicking activity associated with the *rII*⁺ gene product, it would be expected that mixed infection with *rII*⁺-*ligase*⁻ and *rII*-*ligase*⁻ phage should result in decreased phage production. As expected, the

TABLE 1. *Phage production in E. coli B.*

Mutant	Multiplicity of infection	Transmission* coefficient	Percentage† of control burst size
Control (<i>r2-20</i> or <i>r59</i>)	10	1.0	...
<i>amX39</i>	10	0.009	0.01
<i>amX39</i> + 5 min cam‡	7	0.8–1.0	20–40
<i>r59-amX39</i>	10	1.0	36
<i>r2-20 - amX39</i>	10	1.0	38
<i>r2-19 - amX39</i>	10	1.0	38
<i>rb50-amX39</i>	10	1.0	28
<i>amE605</i>	10	0.003	0.01
<i>r70-amE605</i>	10	0.8	41
<i>r2-20 - amE605</i>	10	0.7	35
<i>r59-amX39</i> + <i>amX39</i>	5 + 5	0.08	1.7
<i>r2-20 - amX39</i> + <i>amX39</i>	5 + 5	0.1	1.5
<i>r2-19 - amX39</i> + <i>amX39</i>	5 + 5	0.1	1.6
<i>rb50 - amX39</i> + <i>amX39</i>	5 + 5	0.2	2.2
<i>r48 - amX39</i>	10	0.008	0.01

* See text.

† Burst sizes were calculated as the number of progeny phage per infected cell. The average burst size in the controls was 211 phage per infected bacterium. All values are the average of at least two separate experiments.

‡ Chloramphenicol was added 5 min post infection and removed 30 min later to permit phage maturation.

mixed-infection experiments (Table 1) indicate that the suppressor activity of *rII* mutations is recessive. In all cases where cells are coinfecting with *rII*-ligase⁻ and *rII*⁺-ligase⁻ phage the transmission coefficient and phage yields are considerably reduced. Only about 10 per cent of the mixedly infected cells produce progeny with an average burst size of approximately 1-2 per cent of the control. The addition of chloramphenicol five minutes after infection with a ligase⁻ mutant and its subsequent removal 30 minutes later to allow maturation restores growth to a similar level as does the introduction of an *rII* mutation. For comparison, the results of a "chloramphenicol rescue" experiment are also given in Table 1. It should be noted that the extent of suppression with *rII*-ligase⁻ double mutants is somewhat influenced by the multiplicity of infection. The burst size of cells singly infected with *rII*-ligase⁻ mutants is consistently about 50 per cent lower than is observed when high multiplicities of phage are used.¹¹

In summary, the data in Table 1 indicates that various *rII* mutations when combined with a ligase⁻ *amber* mutation result in high level of growth restoration in the nonpermissive host *E. coli* B, and that the suppressor activity of *rII* mutations is recessive to the *rII*⁺ allele. The lack of suppression by the *r48* (*rI*) mutation suggests that the suppression activity is specific to *rII* mutations.

Reversion studies with rII-ligase⁻ double mutants: In order to show that the suppression of ligase⁻ mutations is indeed due to the *rII* mutations introduced by recombination and not due to some unknown linked gene present in our *rII* stocks, we have examined the growth characteristics of *rII* → *rII*⁺ revertants of several *rII*-ligase⁻ double mutants. Revertants were selected for their restored ability to grow on *E. coli* CR63 (λh) or on *E. coli* K-12 (λh) which are both nonpermissive for *rII* mutants but permissive for ligase *amber* mutants. The plaques appearing on *E. coli* CR63 (λh) or *E. coli* K-12 (λh) were examined for their ability to grow in cells nonpermissive for phage with ligase *amber* mutations. Classification was accomplished by transferring individual plaques to broth and plating for isolated plaques on *E. coli* CR63 (λh) or *E. coli* K-12 (λh). Aliquots of five of these plaques, representing progeny of individual revertants, were then transferred with a sterile toothpick to agar containing a lawn of *E. coli* S/6. Wild-type phage produces a cleared area, ligase⁻ mutants give a negative response, while *rII*-ligase⁻ phage produces an easily recognizable intermediate clearing. Table 2 shows the results of these experiments. With the *r2-19-amX39* double mutant, 64 out of 65 *rII* → *rII*⁺ revertants no longer grew on *E.*

TABLE 2. Growth in *E. coli* B of *rII* → *rII*⁺ revertants of *rII*-ligase⁻ mutants.

Parent phage	Host for reversion	Phenotype of revertant in <i>E. coli</i> B*	
		+	-
<i>r2-19-amX39</i>	K-12 (λh)	0	30
<i>r2-19-amX39</i>	CR63 (λh)	1	34
<i>r59-amX39</i>	K-12 (λh)	22	3
<i>r59-amX39</i>	CR63 (λh)	22	3
<i>rb50-amX39</i>	K-12 (λh)	12	0
<i>rb50-amX39</i>	CR63 (λh)	30	2

* See text.

TABLE 3. *Phage production following infection with some $rII \rightarrow rII^+$ revertants of an $r59 - amX39$ double mutant.*

Strain	Percentage of* control burst size in <i>E. coli</i> B	Transmission coefficient in <i>E. coli</i> B	Percentage of* control burst size in CR63(λ h)
Control (<i>r59</i>)	100	1.0	<.01
Control (<i>amX39</i>)	0.01	0.009	100
<i>r59-amX39</i>	33	1.0	<.01
<i>r59 rev12-amX39</i>	0.9	0.26	74
<i>r59 rev14-amX39</i>	0.009	0.003	61
<i>r59 rev13-amX39</i>	26	0.7	35
<i>r59 rev20-amX39</i>	31	0.9	66

* The average burst size of the controls in *E. coli* B and *E. coli* CR63 (λ h) were 176 and 943, respectively.

coli S/6, thus behaving as nonsuppressed *ligase*⁻ *amber* mutants. However, with the *r59-amX39* and *rb50-amX39* double mutants, most of the revertants still retained the suppressed phenotype.

Table 3 gives the results of burst size and transmission coefficient measurements with a selected group of revertants of the *r59-amX39* double mutant. Although most of the revertant strains isolated from this double mutant are still suppressed (Table 2), there are some which are no longer suppressed, and some which exhibit an intermediate level of suppression. The burst sizes in *E. coli* CR63 (λ h) are generally lower than the control. Similar burst sizes in *E. coli* CR63 (λ h) are observed with a revertant which continues to show suppression (*r59rev20-amX39*) and one which no longer suppresses the *ligase*⁻ mutation (*r59rev14-amX39*).

All of the experiments described indicate that it is specifically an *rII* mutation which removes the stringent requirement for T4-induced *ligase* activity. The isolation of revertants while unexpectedly revealing that the *rII* cistrons are multifunctional, clearly supports this conclusion.

*Net DNA synthesis in rII -*ligase*⁻ double mutants*⁵: Figure 1 shows the levels of DNA synthesis observed after multiple infection of *E. coli* B23 with a *rII*-*ligase*⁻ double mutant and after mixed infection with *rII*-*ligase*⁻ and *rII*⁺-*ligase*⁻ phage. Data is also given for the levels of DNA synthesis after infection with a *ligase*⁻ single mutant with or without the addition of chloramphenicol five minutes post infection. For comparison, the levels of DNA synthesis after infection with wild type are also shown. Nearly normal levels of DNA synthesis are observed when cells are infected with the double mutant and with the *ligase*⁻ single mutant providing chloramphenicol is added five minutes post infection. Cells mixedly infected with *rII*-*ligase*⁻ and *rII*⁺-*ligase*⁻ phage or with *ligase*⁻ phage without chloramphenicol exhibit markedly reduced DNA synthesis.

Physicochemical studies on the integrity of parental DNA: Figure 2 gives the results of sucrose gradient analyses of parental DNA extracted from *E. coli* B23 30 minutes after infection with ³²P-labeled *rII*-*ligase*⁻ phage (left-hand side of figure) or with a mixture of ³²P-labeled *rII*-*ligase*⁻ and *rII*⁺-*ligase*⁻ phage (right-hand side of figure). Parental DNA from cells infected with the double mutant sediments with mature phage DNA and also in a peak which has a D_2/D_1^{12} ratio of approximately 0.6. Behavior on alkaline sucrose gradient sedimentation

(lower left of figure) indicates that the DNA is not extensively nicked. In contrast, when cells are mixedly infected, the DNA is more extensively degraded, and in addition, is highly nicked (lower right of figure). For comparison, sucrose gradient analyses of DNA extracted from cells infected with ligase⁻ phage with or without the addition of chloramphenicol is shown in Figure 3. When chloramphenicol is present, the DNA sediments with mature phage DNA and in a peak having a D_2/D_1 ratio of 0.6 (top left of figure). This DNA is not nicked (lower left of figure). Each of the conditions which prevents parental DNA degradation results in DNA with identical sedimentation behavior and also permits the production of appreciable progeny phage (Table 1).

Physicochemical properties of progeny phage and parent to progeny transfer: Progeny phage from cells infected with an *rII*-ligase⁻ double mutant or a ligase⁻ single mutant when chloramphenicol is added (and removed 30 minutes later to permit maturation) band at the same position as the wild-type reference phage in CsCl (Figure 4, top curves). On neutral sucrose gradients, the DNA of these phage cosediments with reference DNA (*middle curves*). However, on alkaline sucrose gradients the DNA sediments slower than the reference indicating that single-stranded nicks are present. The DNA from *rII*-ligase⁻ phage appears to be somewhat more nicked than that from the ligase⁻ phage. However, in both cases it is possible that the nicking is responsible for the 60–70 per cent decrease in the burst size (Table 1), although the level of DNA synthesis (Fig. 1) is nearly the same as wild type. Since ³²P label was added five minutes after infection, the label

FIG. 1.—Net DNA synthesis in *E. coli* B23. ³²P incorporation into acid insoluble, alkali-resistant material. Infection was done with a multiplicity of 7 phage per bacterium and ³²P (specific activity of 0.05 mc/mg) was present continuously from zero time. Mutants used were *amX39* (*L-r*⁺) and *r59-amX39* (*L-r*⁻).

TABLE 4. Percentage of parental ³²P label to progeny phage.

	Input*	Progeny transfer†
Ligase ⁻ (no cam)	100	0.5
Ligase ⁻ + 5-min cam	100	25–50
<i>rII</i> -ligase ⁻	100	25–50
Wild type	100	50

* Total amount of ³²P-labeled DNA adsorbed to bacteria by 5 min.

† DNase resistant moiety in supernatant upon low-speed centrifugation of Celite filtrate.

FIG. 2.—Neutral (*upper curves*) and alkaline sucrose (*lower curves*) gradient analyses of DNA extracted 30 min after infection of *E. coli* B23 with ^{32}P labeled *r59-amX39* (*left curves*) or equal mixtures of *r59-amX39* and *amX39* (*right curves*). Five to 20% sucrose gradients were used in all experiments. The dashed line is ^3H -labeled reference DNA.

FIG. 3.—Neutral and alkaline sucrose gradient analysis of DNA from *E. coli* B23 infected with ^{32}P -labeled *amX39* with and without the addition of 100 $\mu\text{g}/\text{ml}$ of chloramphenicol 5 min after infection. DNA was extracted 30 min after infection. The dashed line is ^3H -labeled reference DNA.

is associated with newly synthesized DNA and allows a calculation of the viability of the progeny phage. In both cases there appears to be approximately 25 per cent viability of the progeny.

Table 4 shows that there is approximately 25–50 per cent transfer of parental DNA to progeny phage under conditions which cause suppression of ligase⁻ mutations. The recovery of parental label in DNase resistant material is approximately 50–100 per cent of the recovery observed with wild type and 50- to 100-fold greater than is observed with nonsuppressed ligase⁻ infection.

Discussion.—Our results show that the lethality arising from *amber* mutations in the gene (gene 30) which determines the production of polynucleotide ligase of T4D bacteriophage, is reversed when an *rIIA* or *rIIB* mutation is introduced into the same genome. It is very unlikely that *rII* mutation or the addition of

chloramphenicol at five minutes post infection restores ligase function to gene 30 *amber* mutants or permits bacterial ligase to function on phage DNA. Hence, our observations of restored DNA synthesis and viability in the absence of ligase are not in accord with the suggestion of Okazaki *et al.*¹³ that the gene 30 polynucleotide ligase functions *in vivo* to join short stretches of newly synthesized DNA. Our results can best be explained by the assumption that a primary role of phage ligase is to repair DNA nicks resulting from endonuclease action on intracellular phage DNA.²⁻⁴ We suggest that *rII* mutations in some fashion reduce intracellular endonuclease activity and thus permit nearly normal phage development in the absence of ligase function. The levels of restored growth and physico-chemical characteristics of intracellular and progeny DNA obtained with *rII* mutations is strikingly similar to those observed upon suppression of ligase⁻ mutants by the addition of chloramphenicol. In both cases there is appreciable production of progeny phage and no indication of the extensive degradation and nicking of DNA which normally occurs upon infection with ligase⁻ phage. The finding that mixed infection with *rII*⁺-ligase⁻ and *rII*-ligase⁻ phage reduces suppression and results in degraded and nicked DNA also supports our suggestion that the *rII* cistrons control a "nickase" function which degrades DNA in the absence of ligase activity.

Further experiments are necessary to precisely define the mechanism of suppression by *rII* mutations. It is possible that the *rII* cistrons code directly for "nickase" or that they exert an indirect control over nickase activity. When λ lysogens are infected with *rII* mutants there is a decrease in ATP levels¹⁴ and the lethality can be reversed by various changes in the external medium.^{15, 16} It appears that *rII* phage infected cells have altered permeability properties compared to cells infected with wild-type phage.¹⁵ It is conceivable that permeabil-

FIG. 4.—CsCl density gradient and sucrose gradient analyses of progeny phage. The left-hand curves show the results of infection with *amX39*. Chloramphenicol was added 5 min after infection and removed 30 min later. The right-hand curves show the results of infection with *r59-amX39*. In both cases ³²P was added 5 min after infection and remained until lysis at 90 min. The *amX39*-infected cells were resuspended in medium of the same specific activity after removal of chloramphenicol. The dashed line is ³H-labeled reference phage or phage DNA.

ity differences could alter intracellular nickase activity. Our reversion studies with *rII*-ligase⁻ double mutants clearly indicate that the suppression is due to the *rII* cistrons. However, the observation that many *rII* → *rII*⁺ revertants, which have restored growth in λ lysogens, still suppress ligase⁻ mutations (Tables 2 and 3) might indicate a multifunctional role for the *rII* genes. Some preliminary studies¹⁷ indicate that revertants which still suppress ligase⁻ mutants have some of the permeability properties associated with *rII* mutants.

Although the suppression of ligase mutations by *rII* mutations may be explained by altered cellular permeability it is unlikely that suppression by chloramphenicol is due to leakage of intracellular nickase. We have labeled bacteria with ³H-leucine and measured the amount of leakage after phage infection and/or treatment with chloramphenicol. In all experiments, 100 per cent of the pre-labeled bacterial proteins were recovered within cells at times up to 30 minutes post-chloramphenicol addition and/or phage infection.

* This work was supported by National Institutes of Health grant AI 08161 (H. B.), National Science Foundation grant GB 13084 (A. K.) and National Institutes of Health grant CA 10055 (A. K.). The computer evaluation of data was performed in the University of Pennsylvania Medical School Computer Center, supported by National Institutes of Health grant FR 15-06.

† Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218.

‡ Department of Medical Genetics, The University of Pennsylvania, Philadelphia, Pennsylvania 19104.

¹ Weiss, B., and C. C. Richardson, these PROCEEDINGS, **57**, 1021 (1967).

² Kozinski, A. W., *Cold Spring Harb. Symp. Quant. Biol.*, **33**, 375 (1968).

³ Kozinski, A. W., *Biochem. Biophys. Res. Commun.*, **35**, 294 (1969).

⁴ Kozinski, A. W., and M. Mitchell, *J. Virol.*, in press.

⁵ All of the physicochemical experiments were performed with *E. coli* B23. Burst sizes and transmission coefficients were measured and were similar to those observed with *E. coli* B.

⁶ Fareed, G. C., and C. C. Richardson, these PROCEEDINGS, **58**, 665 (1967).

⁷ Berger, H., *Genetics*, **52**, 729 (1965).

⁸ Doermann, A. H., and D. H. Parma, *J. Cell. Phys.*, **70**, supp. 1, 147 (1967).

⁹ Kozinski, A. W., P. B. Kozinski, and R. James, *J. Virol.*, **1**, 758 (1967).

¹⁰ The ratio of the number of infected *E. coli* B capable of producing at least one progeny phage to the total number of infected cells.

¹¹ Although appreciable burst sizes are observed, *rII*-ligase⁻ double mutants exhibit a plating efficiency of less than 0.1 on *E. coli* B or *E. coli* S/6.

¹² Ratio of the distance of the examined aliquot from the top of the gradient to the distance of reference from the top of the gradient.

¹³ Okazaki, R., T. Okazaki, K. Sakabe, K. Sugimoto, R. Kainuma, A. Sugino, and N. Iwatsuki, *Cold Spring Harb. Symp. Quant. Biol.*, **33**, 129 (1968).

¹⁴ Sekiguchi, M., *J. Mol. Biol.*, **16**, 503 (1966).

¹⁵ Garen, A., *Virology*, **14**, 151 (1961).

¹⁶ Buller, C. S., and L. Astrachan, *J. Virol.*, **2**, 298 (1968).

¹⁷ Krisch, H., and H. Berger, unpublished data.