Role of Genetic Recombination in DNA Replication of Bacteriophage Lambda

II. Effect in DNA Replication by Gene Delta

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We have studied the effect of delta mutations in phage lambda on DNA synthesis as assayed by the accumulation of λ DNA in infected cells. We find that delta mutants appear to generate somewhat less DNA than λ^+ in a *rec*⁺ host, suggesting the wild-type delta gene may act in DNA replication. An additional clue to delta function arises if replication is measured in the gamma-negative situation where concatemer formation is abortive. In this situation, the wild-type delta gene has an "inhibitory" effect on replication. A similar inhibitory effect on replication due to delta is observed after infection of P₂ lysogens. We conclude from these studies that the delta gene may act with alpha, beta, and gamma genes, possibly in a process affecting DNA replication.

Normally phage lambda DNA replicates as a circular intermediate during the early period of infection (5 to 20 min) (16). Replication in this mode is characteristically semiconservative (18) and bidirectional (12). Late lambda replication is characterized by neutral sucrose gradients which show a fast-sedimenting form with an estimated size of 2 to 12 monomer lengths (6, 15). Furthermore, replication at late times appears to be asymmetrical, because the parental l-strand sediments in alkaline sucrose as a circular monomer, whereas the r-strand sediments as a linear multimer (8). More recently, Takahashi (personal communication) observed, by using an electron microscope, replication intermediates that appear to be circles with "tails" of several monomer lengths. This evidence is consistent with the rolling circle model for DNA replication (7).

Genetic evidence (18) and biochemical evidence (6) indicate that lambda genes for genetic recombination may act in DNA replication. Genes alpha and beta (5, 13) comprise the Red system, which promotes general recombination. The gamma gene maps next to beta on the genetic map and possibly acts in recombination (18).

Enquist and Skalka (6) found that single mutants defective in beta show reduced rates of DNA synthesis in normal hosts and in the recombination-deficient host *recA*. Replication intermediates during early infection by beta mutants are essentially normal and consist of nicked circles. At late times, however, con-

catemers are appreciably shorter than those found in wild-type lambda infection. This suggests that the recombination genes (alpha and beta) may participate in concatemer formation or propagation. F. Stahl (personal communication) has proposed, for example, that the recombination genes may promote recombination between two circles to form a rolling circle.

The gamma gene of phage lambda is involved in lambda growth, as demonstrated by several criteria: (i) the *Fec* phenotype, in which $\lambda_{red,-gam}$ fails to grow on a *recA* host; (ii) the *Pol* phenotype, in which λ_{gam} fails to grow on a host deficient in DNA polymerase I, and (iii) the *Spi* phenotype, in which $\lambda_{del,red,gam}$ (triple mutant) grows on P₂ lysogens, i.e., is Spi⁻ unlike other lambda mutants or wild-type lambda (Spi⁺; [1]).

The gamma gene may contribute to these phenotypes through some role in DNA replication. Enquist and Skalka (6) found that after infection by *gam* at low multiplicity early DNA synthesis is abnormal and consists of supercoiled and nicked circles. At late times, synthesis of early intermediates continues, and concatemer synthesis fails to increase. The rate of DNA synthesis in λ_{gam} infections, for example, is 30% that of λ^+ .

Biological (18) and biochemical (6) experiments suggest gamma acts in lambda development by interacting with the ATP-dependent DNase which is the product of the *recB* and *recC* genes of the host. The work of Sakaki et al. (11) demonstrating that purified gamma pro-

tein inhibits *recBC* nuclease activity in vitro confirms this possibility. Therefore, in the absence of gamma the *recBC* nuclease is not inhibited and may reduce the accumulation of lambda DNA at late times in infection by preventing the normal formation of concatemers.

Since the lambda genes delta, alpha, beta, and gamma all appear to contribute to the Spi phenotype, it is reasonable to ask whether delta (like alpha, beta, and gamma) has a role in DNA replication.

In this paper, we present evidence that delta is a gene encoding a diffusable product which may function in DNA replication. In the experimental situation where the gamma gene is defective (and concatemer formation is presumably abortive), delta has an "inhibitory effect" on lambda DNA replication.

MATERIALS AND METHODS

Bacterial strains. Media and procedures have been described previously (13), and phage mutants and bacterial strains have been described elsewhere (1). In addition, by conventional techniques we have constructed a P_2 lysogen of AB 1886, which is a *uvrA* derivative of AB1157 and carries the weak amber suppressor, *sup-37*. Experiments with infected *recA* cells employed the strain AB2480 (*recA uvrA*) which is derived from AB1157. HF4704 (P_2) is a P_2 lysogen of *Escherichia coli* C kindly provided by R. Calendar.

Assay of DNA synthesis by λ mutants. Cells of various *E. coli* strains were grown in λ broth to a density of 1.0×10^8 cells/ml. A $0.2\text{-}\mu\text{Ci}$ amount of [^{14}C]thymine per ml (66 mCi/mmol, Schwartz/Mann Co., Orangeburg, N.Y.) was added, and the culture was grown to a density of 2.0×10^8 ml. Cells were centrifuged in 2-ml portions and suspended in TM buffer (0.01 M Tris [pH 7.4], 10^{-3} M MgSO_4). Freshly prepared mitomycin C (Sigma Chemical Co., St. Louis, Mo.) was added to a final concentration of 40 $\mu\text{g/ml}$, and the cells were incubated for 15 min at 37 C in the dark. Alternatively, cells of the *recA* strain AB2480 received 200 ergs of UV light per cm^2 instead of the mitomycin treatment. The treated cells were then centrifuged, washed, and resuspended in lambda TM buffer.

Phage were added to give a total multiplicity of infection of 10 in a typical experiment. Adsorption proceeded 15 min at 37 C and was followed by centrifugation. Cell pellets were resuspended in lambda broth supplemented with 5 μCi of [^3H]thymidine per ml (53 Ci/mmol, Schwartz/Mann Co.).

The experiment was performed at 37 C, and 0.1-ml samples were removed at various times and pipetted onto 2.4-cm 3MM Whatman disks (A. H. Thomas Co., Philadelphia, Pa.). These were placed in 5% trichloroacetic acid (Baker Chemical Co.) and washed repeatedly in 5% trichloroacetic acid, followed by ether-ethanol and ether washes. Acid-precipitable radioactivity was estimated by scintillation counting (Beckman model LS 100) and ^3H counts were normalized against ^{14}C counts.

DNA-DNA hybridization. H514 was grown in a defined medium (AF; as described by Maas [10]) supplemented with 100 μg of L-arginine per ml. The cells were treated with mitomycin C, washed, and after adsorption cells were resuspended in AF supplemented with arginine (100 $\mu\text{g/ml}$) and thymine (3 $\mu\text{g/ml}$). The cells are pulsed with 50 μCi of [^3H]thymidine per ml in either early infection (5 to 10 min) or late infection (35 to 30 min). Incorporation was stopped, and the DNA was prepared by the procedure of Skalka (14). Hybridization was carried out by the method of Denhardt (4) as modified by Skalka (14).

RESULTS

Assay for λ -specific DNA replication. Although phage lambda shuts off host DNA synthesis only partially, it is possible to reduce host DNA synthesis to negligible levels by treatment of a *rec* or *uvr* strain with UV light or mitomycin C. (17). Under these conditions, the program of λ DNA replication is essentially normal, and infection of treated cells thus provides a convenient physiological assay for the effect of various mutations on the accumulation of phage DNA.

Validity of this assay depends upon the specific labeling of phage DNA under conditions of UV or mitomycin treatment. The labeled DNA accumulated in *rec*⁺-infected cells hybridizes to λ DNA with an efficiency of 80 to 90% (Table 1). Conversely, only 4 to 7% of the radioactivity binds to the disks containing *E. coli* DNA. These values agree favorably with those published previously by others.

The data also indicate that DNA labeled early in infection (5 to 10 min) and late in infection (30 to 35 min) is hybridized with approximately equal efficiency.

DNA synthesis by del mutants in the *rec*⁺ host H514. Figure 1 shows the accumulation of lambda-specific DNA for phage mutants defective in gene delta.

In this experiment the host is H514, which is *rec*⁺. Delta single mutants (*delta*₂ and *delta*₄₀₁) appear to accumulate lower amounts of DNA than wild-type lambda, which suggests the delta gene may affect normal DNA replication, even in a *rec*⁺ host.

In Fig. 1 delta mutants are also compared with other lambda mutants defective in general recombination. Cells infected with delta mutants accumulate somewhat more DNA than cells infected with a mutant abnormal for lambda exonuclease (*red*₁; Fig. 1), or beta protein (*red*₁₁₃, data not shown).

Point mutants in gamma generate significantly lower levels of DNA. Cells infected with the *gam*₂₁₀ mutant, for example, accumulate only 25 to 30% the level of cells infected with

TABLE 1. Hybridization of ^3H -pulse-labeled DNA *in vivo*^a

DNA	Input ^b	Bound to λ disk ^b	Hybridized (%)	Bound to <i>E. coli</i> disk ^b	Hybridized (%)	Bound to blank ^b	Corrected (%)
λC_{857} early	8,595	7,779	90.5	380	4.4	137	88.9
$\delta_2\beta_{113}\gamma_8$ early	3,742	3,674	98.2	169	4.5	59	96.6
$\delta_2\gamma_8$ early	4,825	4,221	87.5	335	6.9	181	83.7
δ_2 early	4,939	4,244	85.9	246	5.0	543	84.1
λC_{1857} late	12,890	12,841	99.6	886	6.9	92	95.4
$\delta_2\beta_{113}\gamma_8$ late	5,193	4,429	85.3	340	6.5	319	79.1
$\delta_2\gamma_8$ late	7,321	6,831	93.3	435	5.9	162	91.1
δ_2 late	7,570	7,332	96.9	336	4.4	191	94.3

^a DNA-DNA hybridization. ^3H -pulse-labeled DNA was prepared as described above. Samples of DNA were denatured by boiling and rapidly cooling in ice-NaCl, followed by sonic treatment. Hybridization cocktails contained approximately 0.01 μg of total DNA. A 10- μg sample of denatured λ or *E. coli* DNA was bound to the hybridization discs.

^b Counts per minute.

wild-type lambda (consistent with the result of Enquist and Skalka [6]). Cells infected with the *gam_s* mutant accumulate more DNA than cells infected with *gam₂₁₀*, perhaps because the *gam_s* mutation is "leaky", as has been suggested previously by genetic evidence (18).

DNA synthesis by *del,gam* double mutants in the *rec⁺* host H514. The previous paper in this series (1) describes the construction of new genotypes with a delta point mutation in combination with other mutations. Because the delta gene may act with other genes in this system, clues to the function of delta might be obtained if other genes such as gamma also carry a mutation. Figure 2 shows the program of DNA synthesis by lambda mutants defective in both delta and gamma (*del,gam* double mutants).

Surprisingly, a *del,gam* double mutant accumulates appreciably greater levels of DNA than the *gam_s* single mutant. Whereas the delta mutation by itself decreases DNA synthesis (compare *del₂* and λ^+ , Fig. 2a), the delta mutation in a gamma-negative background increases the levels of DNA synthesis from the low levels seen for the *gam_s* single mutant (compare *del,gam* with *gam_s*). This is observed for the double mutants *del₄₀₁,gam_s* and *del₂,gam_s* (Fig. 2a), and *del₂₀₆,gam_s* (Fig. 2b). The delta mutations shown (*del₂*, *del₄₀₁*, and *del₂₀₆*) appear similar by this assay, although each of the delta mutations was isolated by different methods as described in the preceding paper (1).

This result suggests that the wild-type delta gene in some way "inhibits" DNA replication when gamma is missing. Significantly, the inhibitory effect of the delta gene is most dramatic at a late time, or at the time when concatemer synthesis normally occurs.

Mixed infection by *del,gam* (double mutant) and *gam*. Mixed-infection experiments suggest delta is a gene which encodes an "inhib-

FIG. 1. λ DNA synthesis in mitomycin C-treated *E. coli* H514 *rec⁺ uvr⁻*.

itory factor." In these experiments, cells are infected with two phages differing in delta. One phage has the genotype *del₂,gam_s*, and in single infection this phage by itself makes a relatively higher level of DNA (Fig. 3). A second phage has the genotype *del⁻,gam*, and in single infection it makes lower levels of DNA. The mixed-infection experiment tests whether the *del⁻* phage will inhibit replication of the other phage.

The accumulation of lambda DNA in the mixed infection is at the level of that observed

FIG. 2. λ DNA synthesis in mitomycin C-treated *E. coli* H514 rec^+ at 37 (a) and 39 C (b).

FIG. 3. λ DNA synthesis in mitomycin C-treated *E. coli* H514 rec^+ . The ratio of multiplicity of infection for $\delta_2\gamma_5$ and γ_5 phages in the mixed infection is 4:6.

for the del^+gam phage alone. This suggests the del^+gam_5 phage produces a diffusible factor which inhibits DNA synthesis of the co-infecting del,gam phage.

By this complementation test, the delta mutation is recessive. If the delta mutation actually were an insertion of a new gene or the creation of a new DNA site which stimulates DNA synthesis, such a mutation might be dominant in this complementation test. The result that delta mutations appear to be recessive makes this explanation of delta less tenable and suggests delta is a gene.

This conclusion must be qualified, however, because this complementation test is not defini-

tive proof that the delta product is diffusible. In addition, the interpretation that delta mutations are recessive is deemed most likely only because of the following observation. In repeated experiments, DNA synthesis in the mixed-infection experiments is consistently reduced to a level below the halfway level for the two phages (Fig. 3, the halfway level between gam_5 and del_2,gam_5). Obviously, the interpretation that delta mutations are recessive is not absolute, and other explanations are possible.

DNA synthesis in the $recA$ host, AB2480. Figure 4 shows DNA replication by lambda mutants in the recombination-deficient host ($recA$), AB2480. The results are similar to the results observed in the rec^+ host, H514. In particular, the delta single mutants (del_2 and del_{401}) accumulate lower levels of DNA than wild-type lambda (Fig. 4b), and the del,gam double mutants (del_2,gam_5 and del_{401},gam_5) accumulate higher levels than the single gam_5 mutant (Fig. 4a).

DNA synthesis by λspi in the P_2 lysogen, AB1886. Figure 5 shows the levels of DNA synthesis of phage lambda after infection of the P_2 lysogen AB1886 (P_2). Wild-type lambda makes low levels of DNA in the P_2 lysogen, in agreement with the work of Lindahl et al. (9). The triple mutant $del_{206},beta_{270},gam_{210},CI_{857}$ (which grows on this host) makes higher levels of DNA than λCI_{857} .

The temperature-sensitive delta mutation (del_{206}) present in the triple mutant increases DNA synthesis significantly, because the del^+

derivative of this phage (del^+ , β_{270} , γ_{210} , CI_{857}) in comparison makes significantly less DNA. The experiment is complicated because the β_{270} and γ_{210} mutations are amber mutations which may be suppressed partially by the weak suppressor, sup_{37} , present in the strain AB1886 (P_2). In spite of this possible variable, the delta mutation has a significant effect on DNA synthesis which is consistent with the fact that the delta mutation enables this phage (del_{206} , β_{270} , γ_{210} , CI_{857}) to plate with high efficiency on this host (AB1886; P_2), whereas the del^+ derivative (β_{270} , γ_{210} , CI_{857}) plates with low efficiency.

A complementation experiment (Fig. 5b) suggests the inhibitory effect specific for delta may be mediated by a diffusible factor. In this experiment, cells were infected with two phages (del_{206} , β_{270} , γ_{210} , CI_{857} and β_{270} , γ_{210} ,

CI_{857}). The del^+ phage appears to inhibit replication of the del^- derivative, indicating that the 206 mutation is recessive in this system.

DISCUSSION

Enquist and Skalka (6) have shown that a mutation in the lambda gamma gene sharply reduced DNA synthesis at late times. In λgam -infection of a rec^+ or $recA$ host, concatemer production is abortive and the early circle mode continues into late infection (6).

A delta mutation in combination with a gamma mutation, however, clearly increases the levels of DNA synthesis, particularly after the time when the switch to late synthesis should occur. We surmise that this increased synthesis is attributable to either a production of excess circles (replicating via the early mode)

FIG. 4. λ DNA synthesis in AB2480 $recA^- uvr^-$.

FIG. 5. λ DNA synthesis in AB1886 (P_2) at 39.5°C.

or to a larger number of concatemers. Studies are now in progress to determine the structure of the molecules in the *del,gam* condition to distinguish between these possibilities.

If circle synthesis accounts for the observed increase, we might envision that the delta gene in wild-type lambda normally affects the switch to concatemer formation.

According to one interpretation, the delta gene may serve the lambda replication program by inhibiting the circle mode of replication at late times. Thus, in the situation in which the late mode is blocked by the gamma mutation, delta might have an inhibitory effect on replication through its action on circle production.

According to another interpretation, delta may be a positive factor which normally stimulates the production of concatemers. For example, delta could be an enzyme which nicks the circle and thereby initiates formation of the rolling circle. Hypothetically then the delta protein may shunt circles into a pool of rolling-circle precursors which are attacked by the host *recBC* nuclease, if gamma is not present to control the *recBC* protein. Thus, delta may have an apparent inhibitory effect on the rate of DNA synthesis during late times, if replication is measured in the abortive situation where gamma is absent.

It is also possible that delta mediates an inhibitory effect indirectly. Delta may normally stimulate concatemer formation but function indirectly by inhibiting another protein or factor, which itself prevents concatemer formation. By this model, delta is an "inhibitor of an inhibitor" and thus may not act directly on lambda DNA. Although this model is more complicated, the precedent of gamma (which inhibits the *recBC* protein) suggests this possibility ought to be considered.

Another possibility for delta involves the cell membrane. In this case, the delta gene could code for a protein which promotes the association of lambda DNA to the membrane. Alternatively, delta could be a DNA site necessary for this association to the membrane, and we do not feel the complementation test is sufficiently rigorous to rule this out.

Although we know the delta mutation in combination with gamma and red mutations confers the Spi^- phenotype, the role of these genes in interference in P_2 lysogens appears to be complicated. When λ infects a P_2 lysogen, the DNA circularizes, and transcription of early genes proceeds normally during the first 10 min (9). Lindahl et al. found that replication is initiated but aborted after no more than one

round of synthesis (9). A specific lesion has not been identified, although it has been determined that the partially replicated DNA is not degraded (9).

One possibility is that the P_2 *old* protein together with lambda proteins, red, gamma, and delta, acts at a site(s) to produce the lesion, and this may occur even in a nonreplicating molecule. Mutation in or a deletion of such sites could yield a phage with a *cis*-dominant Spi^- phenotype. F. Van Vliet and J. DeLafonteyne (personal communication) have, in fact, isolated a λ Spi^- mutant bearing a mutation *psi* which may be *cis*-dominant in burst studies in a P_2 lysogen. The genetic nature and map position of *psi* has not yet been reported. It seems unlikely that such sites are involved in a simple restriction phenomenon because there is no evidence of double-stranded cleavage of the λ DNA in a P_2 lysogen (9).

Alternatively, we suggest replication may be required for P_2 interference, and some replicative intermediate may provide the molecular substrate for biochemical event(s) causing abortive growth. This is consistent with the requirement in P_2 interference for λ proteins, red (alpha and beta), gamma, and delta, which affect late replication. The Spi^+ phenotype of λ^+ thus may be associated in some way with the formation of concatemers or precursors to concatemers. According to this hypothesis, λ mutants blocked in concatemer synthesis remove a target of the P_2 *old* protein.

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