The Roles of the Bacteriophage T4 *r* Genes in Lysis Inhibition and Fine-Structure Genetics: A New Perspective

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ABSTRACT

Seldom has the study of a set of genes contributed more to our understanding of molecular genetics than has the characterization of the rapid-lysis genes of bacteriophage T4. For example, T4 *rII* mutants were used to define gene structure and mutagen effects at the molecular level and to help unravel the genetic code. The large-plaque morphology of these mutants reflects a block in expressing *lysis inhibition* (LIN), the ability to delay lysis for several hours in response to sensing external related phages attacking the cell, which is a unique and highly adaptive attribute of the T4 family of phages. However, surprisingly little is known about the mechanism of LIN, or how the various *r* genes affect its expression. Here, we review the extensive old literature about the *r* genes and the lysis process and try to sort out the major players affecting lysis inhibition. We confirm that superinfection can induce lysis inhibition even while infected cells are lysing, suggesting that the signal response is virtually instantaneous and thus probably the result of post-translational regulation. We identify the *rI* gene as ORF *tk.–2*, based on sequence analysis of canonical *rI* mutants. The *rI* gene encodes a peptide of 97 amino acids (M_r = 11.1 kD; pI = 4.8) that probably is secreted into the periplasmic space. This gene is widely conserved among T-even phage. We then present a model for LIN, postulating that *rI* is largely responsible for regulating the gp*t* holin protein in response to superinfection. The evidence suggests that the *rIIA* and *B* genes are not directly involved in lysis inhibition; rather, when they are absent, an alternate pathway for lysis develops which depends on the presence of genes from any of several possible prophages and is not sensitive to lysis inhibition.

ANY volume honoring Jan Drake or dedicated to the topics of mutation and recombination would be incomplete without a discussion of the rapid-lysis mutants of T4, whose role has been central in defining the recombinational and mutational units of heredity, the spectrum of changes caused by various mutagens, and the nature of mutational hotspots. We would further like to dedicate this article to Alfred Hershey, discoverer of the rapid-lysis mutants half a century ago, who passed away last spring, and to Gus Doermann, who first described lysis inhibition after phage infection and who played key roles through many years in supporting our research at Evergreen.

Bacteriophage T4 and its relatives of the T-even family share a unique ability: they are able to control the timing of lysis in response to the relative availability of bacterial hosts in their environment. When *E. coli* are singly infected with T4, they lyse after 25–30 min at 37° in rich media, releasing 100–200 phage per cell. However, when additional T-even phages attack the cell 3 min or more after the initial infection, the cell does not lyse at the normal time (*cf.* Doermann 1948; Abedon 1994). Instead, it continues to make phage for as long as 6 hr, with the exact time of eventual lysis affected by the multiplicity of superinfecting phage and other factors. This delay is termed *lysis inhibition*, or LIN, and appears to contribute significantly to the widespread occurrence in nature of these phage and to their competitive advantage.

One consequence of lysis inhibition is that T-even phages normally form fairly small, rough-edged plaques. As a result of their more rapid lysis, mutants blocked in LIN form sharp-edged, generally larger "r-type" plaques. This makes them easy to identify when plated under appropriate conditions (Hershey 1946). Early recombination experiments mapped such *r*-plaque mutants to three loci named *rI*, *rII*, and *rIII*; the *rII* locus actually consists of two neighboring genes, *rIIA* and *rIIB*. These rapid-lysis mutants played important roles in constructing early maps of T2 and T4 and in determining the genetic circularity of the map (Streisinger *et al.* 1964). Mutants defective in the three loci can be distinguished by their plating behavior on different bacteria, as well as by recombination. Mutants altered in *rI* form r-type plaques on K strains of *E. coli*, in addition to the B strains where they were first studied (Benzer 1957). In contrast, mutants defective in *rIIA* or *rIIB* form wild-type plaques on non-lysogenic K strains; it thus appears that for some reason the *rII* gene products are necessary

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to observe lysis inhibition on B, but on K they are not needed to establish LIN. This has the experimental advantage that *rII* mutants can be grown on the latter strains without experiencing any selective disadvantage. In addition to their defect in lysis inhibition, Benzer (1957) found that phage mutated in *rIIA* or *rIIB* have a second genetically useful property: they do not form plaques on strains of K-12 lysogenic for phage λ. Revertants or wild-type recombinants can generally be detected to a frequency of 10^{-8}. The factors blocking *rII* mutants from plating on λ lysogens have been extensively studied, as reviewed by Snyder and Kaufmann (1994). Whether there is any connection between the *rII* mutants' defect in establishing LIN on B and their lack of plating on λ lysogens remains unclear: this is discussed further below, with a rather surprising conclusion.

Benzer eventually used hundreds of independently isolated *rII* mutants to determine the fine structure of the gene to the nucleotide level (*cf.* Benzer 1955, 1966). Most of these mutants were able to revert to wild type at measurable rates and thus were considered to carry *point* mutations (many of which turned out to be small frameshifts). However, many mutants would not revert and recombine with two or more point mutations at different sites; it was later confirmed that they involved deletions of a *sequence* of bases. Because no two overlapping deletions can recombine, Benzer used these properties to arrange a set of 145 deletions in an unambiguous sequence. This set of deletions can then be used to map any mutation by first using long deletions to localize it roughly, then crossing it against shorter deletions to map it more precisely. Standard crosses can then determine its position relative to other nearby point mutations. Benzer thus determined that the *rII* point mutations map at many sites, some so close together, he concluded, that they must be within one or two nucleotide pairs of each other. No genes in any other organism have been mapped so thoroughly.

The spectrum of spontaneous mutations showed mutation frequencies varying by orders of magnitude, including two "hot spots" with very high mutation rates. The well-defined *rII* system was also used to look at the mutational spectra of various mutagens, demonstrating that different mutagens have very different patterns and, in many cases, identifying the specific kinds of changes. The power of the *rII* system in mutational studies is further reflected in the fact that T-even phage have been used to test over 300 substances for potential mutagenicity; Drake and Ripley (1994) included a complete list of agents that had been tested, with references and results obtained. As emphasized by Drake and Ripley (1994), the *rII* system remains one of the few genetic systems that can screen mutations efficiently in the forward direction (using plaque morphology) and also select mutations in the reverse direction, from mutant to wild type (using hosts lysogenic for λ).

The *rII* proteins seem to have a wide and confusing range of functions. The *rIIA* and *B* genes both encode proteins found associated with the membrane (Weintraub and Frankel 1972; Ennis and Kievitt 1973; Huang 1975). They are essential for T4 recombination (and thus for late replication) in λ-lysogenic hosts, and may be involved in anchoring the replication/recombination complex to the membrane (Mosig *et al.* 1984; Mosig 1994). Mutations in *rIIA* or *B* partially suppress mutations in two genes involved in recombination, replication, and DNA packaging: 32 (the single-stranded DNA-binding protein) (Krylov 1973; Mosig *et al.* 1984; Mosig 1987) and 30 (ligase) (Berger and Kozinski 1969; Karam 1969; Ebisuzaki and Campbell 1969). However, they also make a mutational defect in gene 49 (DNA packaging) more severe (Mosig *et al.* 1984). Mutations in these recombination-associated genes, in turn, suppress the *r*-type plaque morphology of *rII* mutants (Mosig 1987). It appears that in rII^+ infection, the host ligase is somehow sequestered and relatively unavailable to substitute for a defective phage ligase (Mosig and Breschkin 1975). There seem to be so many complex interactions that Mosig (1987) suggested that the *rII* genes be given at least honorary status as recombination genes, even though *rII* mutations are not known to affect recombination.

The suppression of ligase mutations by *rII* mutations may at least in part be related to less discriminate DNA packaging for *rII* phage in *E. coli* B (Carlson and Kozinski 1969; K. Carlson, personal communication). Normally, DNA packaging in T4 involves threading somewhat more than one genome equivalent into the head from a very large, multi-branched replicating complex, mending nicks and cutting off branches in the process. However, *rII* and *rI* mutants are uniquely able to package unit-size DNA and smaller fragments and even unreplicated parental DNA. After infection with a T4 ligase mutant, host ligase and other repair enzymes may only partially replace the missing phage enzyme, resulting in phage DNA where most DNA strands are less than unit length, and all molecules unit length or longer contain discontinuities in the form of gaps (Carlson *et al.* 1973). Somehow, the *rII* mutations permit maturation of such DNA and, thus, the production of enough progeny phage to make a plaque. (Carlson and colleagues note that this ability to mature short or gapped DNA is also shared by *rI*, but not *rIII*, mutants; the implications of this observation have not been studied further.)

It is ironic that we do not yet have a better understanding of the process of lysis inhibition or of the ways in which the various T4 rapid-lysis mutants block its implementation, even though these mutants were among the first identified in T4. Furthermore, at the genetic level, the T4 *rIIA* and *B* genes are better understood than any other gene in any organism. Many would agree with Snyder and Kaufmann's (1994) statement

that "the status of the *rII* genes of T4 in the history of genetics compares to that of the gene causing Mendel's rough and smooth peas," having played key roles in our understanding of the molecular basis of mutation and recombination (Benzer 1957), the mechanisms of action of a wide variety of mutagens (Drake and Ripley 1994), and the triplet nature of the genetic code (Crick *et al.* 1961).

We know that the usual process of lysis of T4-infected cells involves a lysozyme encoded by gene *e* whose access to the peptidoglycan layer is normally blocked until ~25–30 min after infection under standard lab conditions. Lysozyme egress is then mediated by the product of gene *t* (Josslin 1970, 1971), also called *stII* (Krylov and Plotnicova 1972; Krylov and Yankovsky 1975). Lysis inhibition delays this *t*-dependent lysis, while energy poisons can cause lysis to occur prematurely. However, we still have no knowledge of the mechanism of the normal lysis clock or of the process of LIN. As discussed by Young (1992), "the entire *t*-mediated, *r*-controlled lysis phenomenon remains shrouded in mystery, all the more vexing in light of how long this event has been available for genetic analysis and also, perhaps more embarrassingly, how intimately related it is to some of the definitive and founding experiments of molecular genetics." Here, we take a step toward unraveling that mystery by integrating recent evidence related to the molecular players involved in lysis inhibition. This includes identifying the *rI* gene and some characterization of its product, as well as an analysis of information related to genes *rII* and *t*. Somewhat heretically, we suggest that *rII* may not actually be related to lysis inhibition at all. The evidence suggests that the absence of the *rII* proteins may, on some strains, simply lead to cell breakdown prior to and independent of the action of the normal LIN-sensitive clock, releasing enough progeny to make plaques. In this context, we present a model in which *rI* is largely responsible for regulating the functional state of *t* in response to superinfecting phage and thus for establishing lysis inhibition.

MATERIALS AND METHODS

Strains: T4D, T2, and T6 and the *rI* mutants *r*48, *r*52, *r*53, *r*57, and *r*58 are from the collection of A. H. Doermann, which was moved to Evergreen at his retirement. Proflavine-induced *r* mutants and the putative *rI amber* mutants are from Leslie Smith and Jan Drake, as are *rII* frameshift mutant FC0, *amber* mutants *r*EM64 and *r*EM84 and Krylov's original *rV* mutant; *rII* deletion mutants *r*18F, *r*196, *r*H23, and *r*H88 are from Harris Bernstein, as are Abedon's T4D and *am*N91(37). The RB family of phages, described by Russell and Huskey (1974), was obtained from the T4 collection curated by Bill Wood at University of Colorado, Boulder. C16, Poland and Tbilisi phages came from Liana Gachechiladze of the Bacteriophage Institute, Tbilisi, Georgia. T7 was the gift of Ian Molineaux, University of Texas.

Bacterial strains: The *E. coli* B and K-12 (Sup^0) used by Abedon for the lysis curves and the CR63 (*supD*) for growing phage stocks were gifts of Jan Drake, while Abedon's *E. coli* S/6/5 came from Harris Bernstein. At Evergreen State College *amber* mutants were grown on K803 (*supE*), received from Larry Snyder in 1975. Most experiments were carried out on B from our collection.

Media: M9 medium, Hershey broth (HB), and phage diluent were made as described by Carlson and Miller (1994). For Hershey agar, Bacto-peptone was substituted for Tryptone, and only 12 g agar/liter was used (6.5 g for top agar). T4-TSB is trypticase soy broth to which 2.9 g/liter NaCl has been added.

Complementation and recombination studies: Complementation between various putative *rI* mutant phages was analyzed by mixing the two phages at equal concentrations in small flasks, adding 2–5 ml of exponential-phase *E. coli* B in HB to give a moi of 5–10 of each phage, incubating with aeration at 37°, and scoring the time of spontaneous lysis. Infection with the single mutants was used as the control. The test was considered to show no complementation for the *r* phenotype if spontaneous lysis still occurred between 30 and 35 min despite superinfection; in some cases, intermediate results were obtained that were hard to interpret, with lysis occurring gradually over 30–50 min.

With some of the putative *r* mutants, we had difficulties seeing clear enough differences in plaque morphologies to carry out clear-cut recombination studies. Under our conditions, the putative *rI amber* mutants made very small though sharp-edged plaques; it was not easy to count with certainty a small number of fuzzy-edged, wild-type plaques among them that would have come from recombination. However, it appeared that the recombinant frequency was on the order of 1–2% between any two putative *rI* mutants.

PCR and DNA sequencing: At Evergreen, DNA sequencing of various *rI* and *t-amber* mutants was carried out from PCR products generated in a Stratagene (La Jolla, CA) RoboCycler using purified phage stocks as the templates. Primers were selected using the sequence of the region that we had already determined (M. Mzhavia, E. Marusich, T. Djarachishvili and E. Kutter, unpublished results), using the constraints of a genome that is two-thirds A-T. The resultant bands were purified for sequencing using the Promega (Madison, WI) Wizard gel extraction kit. Sequencing was carried out by hand using the Promega Femtomole Sequencing Kit. At NIEHS, DNA sequencing was carried out on an ABI 377 (for the rp mutants and *rI*-20) from PCR products purified using the QIAquick PCR purification kit from Qiagen (Santa Clarita, CA). Each mutation was confirmed by sequence analysis in both directions, from multiple PCR products. The primer sequences used were as follows: rI 5′ = GTTAAGGCCGTGCA TCG; rI 3′ = CCTAAGTATTCATCTGCCTTTC; r3 5′ = GCT ATTCCGTGTCTTTATAAGTC; rI 3′ = CTTCAGTGTTACCA CAAAGTGACG.

Lysis profile experiments: Lysis experiments were carried out by infecting cells for one generation (moi = 5) or for two generations (moi = 0.1). For moi > 1, infections were synchronized by infecting washed *E. coli* in M9 salts solution in the absence of glucose; after a 15-min adsorption period, phage multiplication was initiated by the addition of glucose. Turbidity measurements were generally made using a Klett colorimeter (Klett Manufacturing Co., Inc., NY). Superinfecting phage were added at appointed times in M9 salts; controls were given the same volume of M9 salts without superinfecting phage. At high cell densities, T-even phages from the first infected cells to lyse adsorb onto surrounding cells and induce LIN (Doermann 1948; Abedon 1992). To permit densitometric observations of normal lysis, we therefore used as our wild-type "unsuperinfected" control a tail-fiber mutant, *am*N91(37) on non-*amber*-suppressing host strains (*cf.* Kao and

McClain 1980). Those experiments involving two-step lysis profiles were carried out in a lidless, flat-bottom 96-well microplate (Corning #25880-96, Corning, Inc., Corning, NY) shaken at 37°. Here, phage were added directly to growing cells at an moi of ~0.1 and followed for two generations. Absorbance readings at 550 nm were taken every 30 sec using a SpectraMax tunable microplate reader (Molecular Devices, Sunnyvale, CA).

RESULTS

ORF *tk.−2* is the *rI* gene: While the *rIIA* and *rIIB* cistrons were mapped in detail early on and define the zero point of the circular T4 genomic map, the locations of *rI* and *rIII* proved more difficult to determine precisely. The *rIII* gene was identified as ORF 30.10 by Raudonikiene and Nivinkas (1992). The main keys to the location of *rI* were given by deletion mutants, because it lies in the middle of an 18-kb region that can be deleted without affecting viability of the phage under lab conditions. This region contains 39 open reading frames (ORFs) that are probably expressed, only eight of which have been characterized with regard to function. There were no nearby essential genes to facilitate precise positioning of *rI*. A set of deletion mutants isolated as being folate-analogue resistant due to their lack of a *tk* gene all showed a rapid-lysis phenotype generally attributed to an *rI* defect (Johnson and Hall 1973). Wilson (1976) developed a technique to isolate new deletion mutations and determined their map positions through heteroduplex mapping. His data indicated that *rI* was probably 6 kb further from the *rIIA*/*rIIB* junction than previously thought, at ~59 kb.

We therefore sequenced the region between position 56.4 kb and *tk* (59.7 kb) in the canonical *rI* mutant, *r*48 (Doermann 1952), and in two hydroxylamine-induced supposed *rI amber* mutants, *am*47-22 and *am*48-23. This region contains 11 uncharacterized ORFs that appear likely to be expressed. We found a change in ORF *tk.−2* in the *r*48 strain: a deletion of one adenine in a run of five between base pairs 193 and 197 (Figure 1). This change was verified using three separate PCR products. Doermann's other four *rI* mutants (*r*52, *r*53, *r*57, and *r*58) were then tested and also showed alterations in *tk.−2* (Figure 1), which we therefore designate as the *rI* gene. In addition, eight new proflavine-induced mutants with *r* plaque morphology on K-12 are *rI* mutants, as indicated in Figure 1.

The newly-identified *rI* gene has no codons convertible by single-base transitions to an *amber* codon, and no mutations in *tk.−2* (or in the entire 3.3-kb region sequenced) were found in either *am*47-22 or *am*48-23. This was surprising because both of these mutants have *r* plaque morphology, appeared to give less than 1% recombination with known *rI* mutants, and did not complement our known *rI* mutants or each other to give LIN in liquid-culture lysis tests. The explanation became obvious when liquid-culture lysis tests were carried out by coinfecting *E. coli* B with these putative *rI amber* mutants and wild type.

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T4          G insert=rp25, CC insert=rp37
mutants     ↓
       1 ATG GCC TTA AAA GCA ACA GCA CTT TTT GCC ATG CTA GGA TTG TCA TTT GTT TTA TCT CCA TCG ATT GAA GCG
T4     1 Met Ala Leu Lys Ala Thr Ala Leu Phe Ala Met Leu Gly Leu Ser Phe Val Leu Ser Pro Ser Ile Glu Ala
T2                               Val

T4                                  AT insert=r53,r58
mutants                             ↓
      73 AAT GTC GAT CCT CAT TTT GAT AAA TTT ATG GAA TCT GGT ATT AGG CAC GTT TAT ATG CTT TTT GAA AAT AAA
T4    25 Asn Val Asp Pro His Phe Asp Lys Phe Met Glu Ser Gly Ile Arg His Val Tyr Met Leu Phe Glu Asn Lys
T2                                                                              Thr

T4                                                                  A delete=r48,r52,rI-20
mutants                                                             ↑
     145 AGC GTA GAA TCG TCT GAA CAA TTC TAT AGT TTT ATG AGA ACG ACC TAT AAA AAT GAC CCG TGC TCT TCT GAT
T4    49 Ser Val Glu Ser Ser Glu Gln Phe Tyr Ser Phe Met Arg Thr Thr Tyr Lys Asn Asp Pro Cys Ser Ser Asp
T2

                                                                  C insert=rp31, CA insert=rp77
T4                          C=r57                  rp19,rp28=insert G        ATT delete=rp10
mutants                     ↑                      ↓                ↓        ↑
     217 TTT GAA TGT ATA GAG CGA GGC GCG GAG ATG GCA CAA TCA TAC GCT AGA ATT ATG AAC ATT AAA TTG GAG ACT
T4    73 Phe Glu Cys Ile Glu Arg Gly Ala Glu Met Ala Gln Ser Tyr Ala Arg Ile Met Asn Ile Lys Leu Glu Thr
T2                                                                              Lys

T4
mutants
     289 GAA TGA
T4    97 Glu ***
T2
```

Figure 1.—Sequence of the T4 *rI* gene. Changes identified in various mutants are indicated above the sequence; differences between T2 and T4 *rI* genes are shown below it.

Both mutants have a dominant phenotype; the infected cultures lysed 30–40 min after infection, rather than showing extended lysis inhibition. Because they appear to map close to *rI*, we suggest that they may affect one of the unidentified ORFs lying between *tk* and the *tRNA* region. Krylov and Yankovsky (1975) reported evidence for another gene, *stIII*, mutations in which arise as pseudorevertants of tight mutations of gene *t*. They report that the stIII phenotype is dominant; in liquid culture, phage are gradually released beginning about halfway through the lytic cycle, even when the cells are coinfected with $stIII^+$ phage. They mapped this gene to the general vicinity of *rI*, and the phenotypes resemble those of these putative *rI amber* mutants. Unfortunately, the original *stIII* mutants are no longer available.

The *rI* gene encodes a small protein that is predicted to be secreted to the periplasmic space: The *rI* gene encodes a peptide of 97 amino acids with a molecular weight of 11.1 kD and pI of 4.8. The hydrophobicity predictions for gp*rI* are shown in Figure 2, compared with those for gp*rIII*, which is quite hydrophilic. Notably, the predicted *rI* peptide contains an N-terminal hydrophobic domain of 16 amino acids. Computational analyses kindly carried out by Dana Boyd, using a new method discussed by Boyd *et al.* (1998), predict that *rI* is secreted, rather than being a membrane protein. This prediction was also made by the Danish SignalP web site (http://www.cbs.dtu.dk.services/SignalP/), which predicts a signal-peptide cleavage site between position 24 and 25. The C terminus region appears to be important for function. For example, mutation *rp10* involves the in-frame deletion of codon 92 (Ile), only five amino acids from the stop. Mutations *rp*19, *rp*28, *rp*31, and *rp*77 result in frameshifts within eight amino acids of the end. The only observed missense mutation (*r57*) changes 78 Arg to Gly.

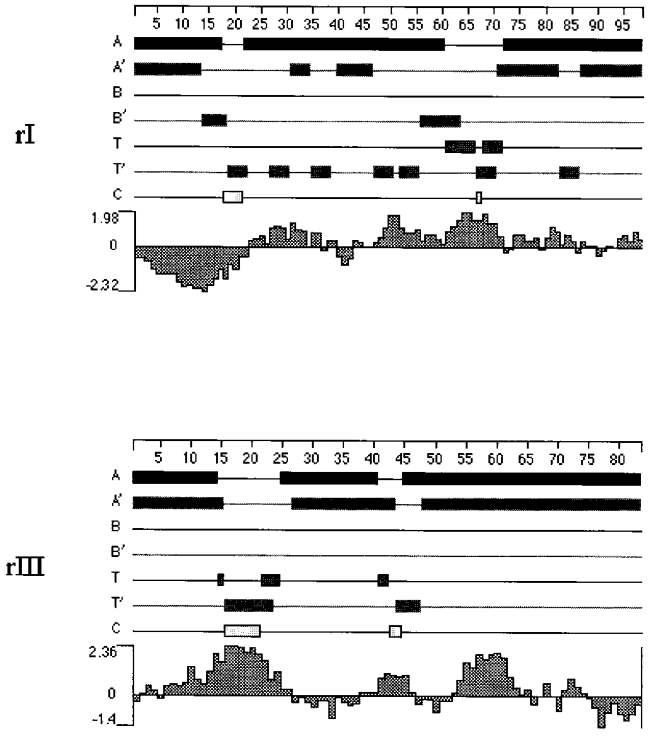

Figure 2.—Hydrophilicity plot predicted for the *rI* and *rIII* gene products (by the Kyte-Doolittle algorithm), as displayed by the DNA Protean program. The amino acid position is indicated along the top.

***rI* and *rIII* served by both early and late promoters:** The *rI* gene is the second gene in a three-ORF transcription unit, defined by a canonical late promoter between *tk* and *rI.1* and a terminator after *rI.–1* (Figure 3). The *rI* gene also lies 3.3 kb downstream of an early promoter, with no intervening terminator. Preliminary RT-PCR data confirm *rI* expression from both promoters (data not shown). Early expression is not surprising, because lysis inhibition can be induced when secondary phage adsorption occurs as early as 3 min after infection (Doermann 1948; Rutberg and Rutberg 1965).

The *rIII* gene is similarly regulated; it is the first gene in a two-ORF transcription unit flanked by a late promoter and a terminator (Raudonikiene and Nivinskas 1992). In addition, early and middle transcripts of *rIII* would arise from two upstream promoters without intervening terminators, one of them middle-mode (−483 bp) and one early (−2.3 kb) (Figure 3).

Most T4 late genes are transcribed in the clockwise direction of the genomic map, while all known genes expressed early in infection are transcribed counterclockwise. The few late genes transcribed counterclockwise are generally included on (extended) early, as well as (short) late transcripts. In the cases of lysozyme (*e*), a small outer-capsid protein (*soc*), and the intron-encoded homing endonucleases, an RNA stem-loop blocks translation of the early transcripts (Miller *et al.* 1994). In each case, the late promoter is in the middle of the potential stem-loop region, so translation proceeds unimpeded from the late transcripts. Neither *rI* nor *rIII* early transcripts have such stem-loops and both appear likely to be translated early as well as late in infection, as expected.

Mutation of *rI.−1* does not generate a rapid-lysis phenotype: In T4, genes grouped together into transcription units often have related functions. Examples include clusters of DNA replication, host-shutoff, baseplate, head, tail, and tail-fiber genes. This raises the question of whether *rI.1*, *rI.−1* and/or *rIII.−1* might also be related to lysis and/or to lysis inhibition. During sequencing of the various mutants in the extended *rI* search, we found a mutation in our lab stock of T4D that inserted a G into the sequence GGT-GAG in *tk.−3*, resulting in GGG-TGA. Correcting this problem merged our original ORFs *tk.−3* and *tk.−4* (*cf.* Kutter *et al.* 1994) into one reading frame. This new ORF, designated *rI.−1*, encodes a hydrophilic protein with 129 amino

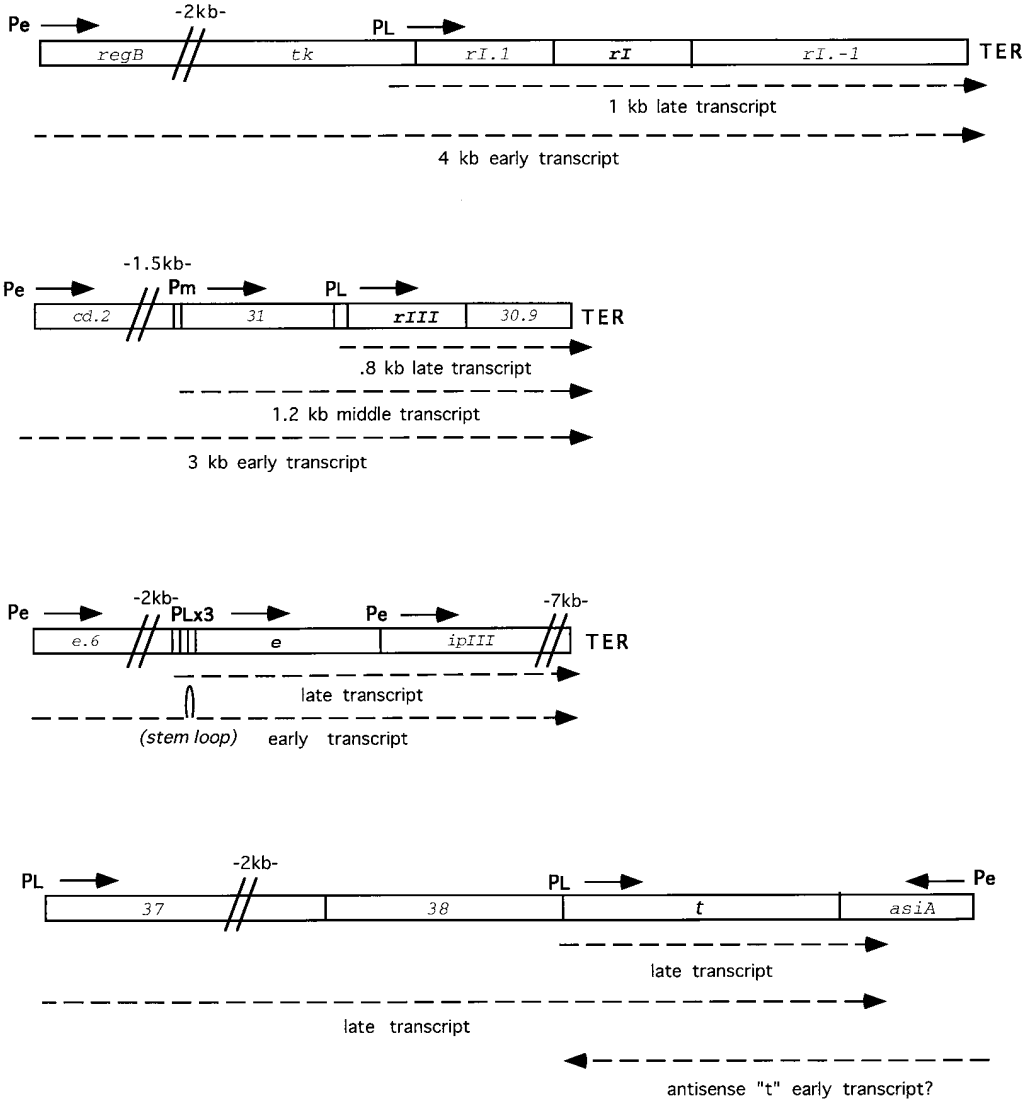

Figure 3.—Gene arrangement and patterns of transcriptional regulation predicted for genes *rI*, *rIII*, *e*, and *t*. Note that transcripts containing *rI*, *rIII*, *t*, and antisense *t* are predicted from analysis of genome, while gene *e* late and early transcripts have been shown previously (McPheeters *et al.* 1986). Antisense *t* mRNA should be produced because there is no terminator between *t* and *asiA*; albeit the physiological relevance has yet to be determined.

acids (M_r = 14.6 kD) and a pI of 5.6. Despite the inadvertent mutation, which truncates *rI.−1* after 60 amino acids in our old "wild-type" lab strain, we observed normal plaque morphology and lysis kinetics in infected cultures. It therefore appears that gene *rI.−1* is not required for lysis inhibition, although it may still play some supportive lysis-related role.

Conservation of the *rI* and *rIII* genes among the T-even family of phages: The family of T-even phages is large and diverse, with members able to grow in all of the enteric bacteria and some of their more distant gram-negative relatives (*cf.* Ackermann and Krisch 1997). The general plaque morphology of T-even phages and the usefulness of lysis inhibition make it seem likely that LIN is widely conserved. To check the degree of conservation of the *rI* gene, we first sequenced *rl* from T2; the few differences from T4 are indicated in Figure 1. We also tested a variety of T-even phages for the production of an appropriately sized PCR band using primers that flank *rI* and are internal to *rI.I.* and *rI.−1*. To date, all T-even phage tested produce the appropriately sized PCR band; those tested multiple times include T2, T6, Tibilisi, Poland, C16, LZ4, LZ5, RB5, RB6, RB7, RB9, RB10, RB12, RB49, and RB69, although it was always faint in the latter two. Further sequencing will clearly be needed to determine the interrelationships and the extent of conservation, The result with these primers suggests that not only *rI* itself but the entire operon is conserved.

The *rIII* gene also appears to be quite widely conserved. In this case, the primers used were within *rIII*. An appropriately sized band was seen for most of the phages tested (T2, T6, LZ4, LZ5, Poland, and RB69), but not for RB49. The latter has been classified as a "pseudo-T-even" phage, with the structural proteins in common but apparently vast changes in other regions; it also uses C rather than HMC in its DNA (Monod *et al.* 1996). It will be interesting to determine whether RB49 actually carries no *rIII* gene or whether the sequence has simply diverged so much that these primers (based on the T4 sequence) do not bind.

Phage T7, used as a control, showed no band for *rI*

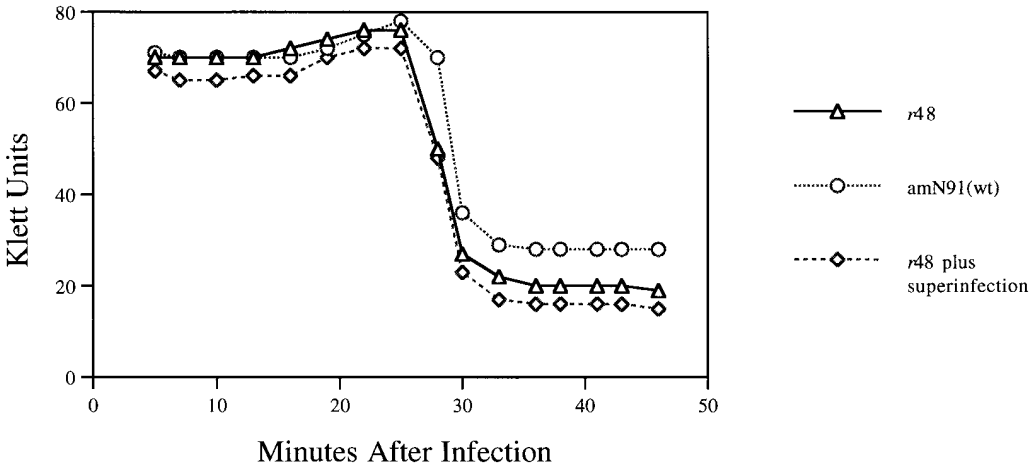

Figure 4.—Lysis profiles of *rI* mutant r48 without (△) and with (◇) superinfection, compared with gene 37 mutant *am*N91 (○), which shows the wild-type pattern in the absence of lysis inhibition.

or *rIII*, as was expected because T7 and its relatives show no lysis inhibition (Young 1992).

Lysis profiles of cells infected with *r* mutants: Doermann (1948) demonstrated the rapid-lysis phenotypes of *rI*, *rII*, and *rIII* mutants in single-step growth curves. We further characterized the lysis profiles of *rI* and *rII* by using as our "wild-type" control a tail-fiber *amber* mutant and carrying out the experiment in su^- hosts. Under these conditions, progeny phage cannot adsorb, permitting densitometric observation of normal lysis with no induction of LIN. Additionally, we employed a 15-min preadsorption in the absence of a carbon source to improve synchronization. Densitometric lysis profiles of B or S/6/5 infected with *r48* (*rI*), *rFC0* (*rII*) or *am*N91(37) are presented in Figures 4 and 5. The pattern for the *rI* mutant in the experiment presented in Figure 4 is very similar to the *am*N91 "wild-type" pattern. Thus, the *rI* mutant displays lysis kinetics that are very similar to those in non-lysis-inhibited r^+ phage. We conclude, therefore, that *rI* phage display the wild-type lysis kinetics observed in the absence of lysis inhibition. In contrast, we notice that *rII* mutant lysis starts somewhat earlier and occurs more slowly than the lysis of the *rI* mutant. This difference is particularly apparent in a two-cycle comparison between *rII* and *rI* (Figure 5). We conclude that the *rII* mutant displays altered normal lysis, as well as not expressing LIN on this B strain of *E. coli*.

Induction of lysis inhibition after initiation of lysis in liquid cultures: The data of Mukai *et al.* (1967) suggest that LIN can be induced even after lysis starts. To confirm and explore these results, we superinfected cultures of K-12 infected with g37(*am*N91) at various times as they were beginning to lyse (Figure 6). Superinfection at an moi of 10 was carried out every 3 min from 30 to 45 min after primary infection. Without superinfection, the culture began lysing after 30 min (Klett > 15) and finished between 43 and 48 min (Klett = 5). Superinfecting cultures prior to 36 min—while the turbidity was dropping—consistently caused an almost immediate stabilization of the optical density; this lysis inhibition was maintained for at least 3 hr.

DISCUSSION

One of the longest standing mysteries of T4 biology is the mechanism of lysis inhibition and the function of the *r* genes (*cf.* Alberts, 1994). The T4 family of phages appears to stand alone as the only lytic virus capable of significantly modifying its growth cycle in response to an outside signal which would otherwise be

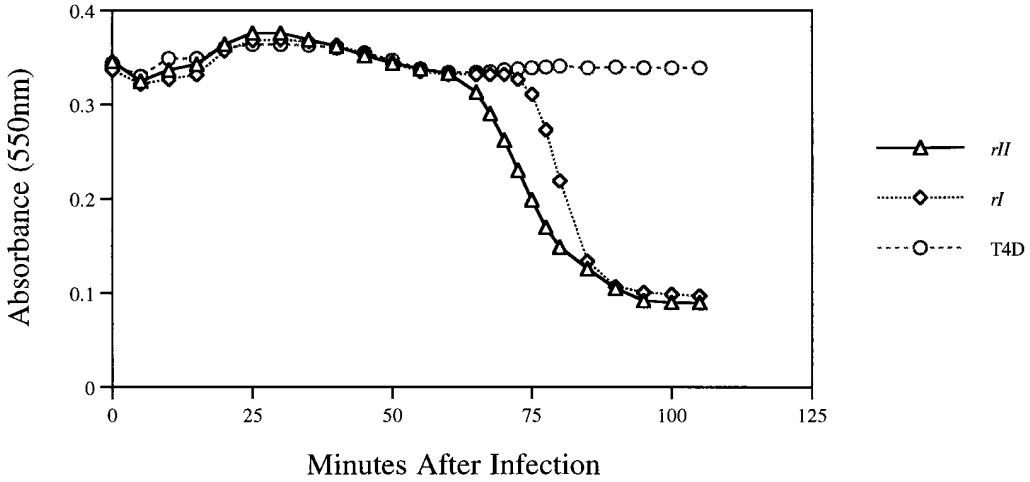

Figure 5.—Patterns of lysis of T4D (○), *rI* mutant r48 (◇), and *rII* mutant rFC0 (△) after infection at low moi, giving two cycles of infection. Note that the *rII* mutant lyses earlier and more gradually; exactly this same pattern has been seen with a number of different *rII* mutants, both point mutants and deletions.

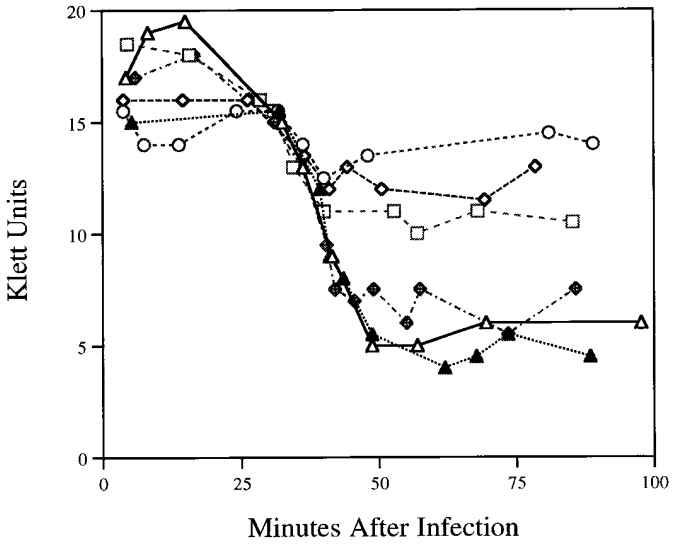

Figure 6.—Induction of lysis inhibition in liquid culture at high density after some of the infected cells have started to lyse. Cultures of K-12 infected with *am*N91 were superinfected at moi = 10 at times shown. Two nonsuperinfected controls are shown to account for variability in initial culture density.

innocuous to its growth. In a way, LIN induction can be viewed as a ''dense conditions check point.'' Between 3 min after infection and the actual start of lysis, the question is continually asked, ''Are there external T-even phages in the immediate environment?'' If no, then lyse at the end of a specified normal latent period; if yes, than induce extended lysis inhibition.

Virtually no insight, however, has been gained as to the nature of the signal for lysis inhibition and the functions of the genes mutations in which block expression of lysis inhibition. Much effort has been put into studying *rIIA* and *B* with few results relevant to LIN (Young 1992). The other reported *r* genes (*rI*, *rIII*, *rIV* and *rV*) have been studied far less than *rII*. No conditions have been found under which their lack is lethal, and few or no lethal genes lie nearby. A major purpose of this paper is to identify and characterize the major players involved in lysis inhibition and to suggest the outlines of a model. As discussed below, the data suggest that lysis inhibition involves functional regulation of a holin directed by gene *t* through the action of the product of gene *rI;* other genes may also participate. However, we conclude that the *rII* gene products are not directly involved in lysis inhibition, but rather that their absence results in cell lysis when certain prophages are present, and that this occurs by some mechanism that superinfecting phage are not capable of blocking.

What is the role of gene *t* in lysis and lysis inhibition? The product of gene *t* probably forms the holin that conducts the lysozyme (gp*e*) into the periplasmic space, even though the protein looks very different from all the other known holins, as discussed by Young (1992). The evidence for this includes the fact that the cloned *t* gene can substitute for the λ *S* holin gene (Lu and Henning 1992). Also, mutations in gene *t* block the lysis and energetic effects (Josslin 1970, 1971). We have determined that the putative *amber t* mutants studied by Riede (1987) do indeed have the appropriate alterations in the gene identified as *t* (K. Gailbreath, unpublished results). We speculate that the unusual structure of the *t* holin is related to its ability to undergo lysis inhibition. While it does not have the second apparent membrane-spanning hydrophobic alpha helix, it does have a potential amphipathic alpha helix that could be involved in assembling a membrane channel (Lu and Henning 1992). Thus, the *t* protein is the most probable target of regulation when excess phage are sensed during infection (Young 1992).

Krylov and Zapadnaya (1965) reported isolating *ts* mutants that defined two new apparent rapid-lysis genes, *rIV* and *rV*, neither of which has yet been located precisely. Mutants in *rV* are identical in phenotype to *rI* in that they form large, sharp-edged plaques on all host strains tested. However, they map at the opposite side of the genome, in the general vicinity of genes *38*, *t*, and *motA* (Krylov and Yankovsky, 1975; J. Drake, unpublished results). It now appears likely that *rV* is actually *t*. Krylov's original *rV* mutant was sequenced in the region from 160.2 kD (end of tail gene *38*) to 163 kD (*motA*), and the only difference from the wild-type sequence was a substitution of lysine for arginine near the start of gene *t*. Several *rV* mutants isolated in the Drake lab also show point mutations in *t* (H. K. Dressman and J. Drake, unpublished results). Perhaps the rV phenotype reflects a particular region of *t* that interacts with the lysis-inhibition signal; our model would predict that such mutants would have the same set of phenotypes as *rI*.

It has been suggested that *rIV* may be equivalent to a yet unidentified gene called *spackle* (*sp*), mutations of which accumulate in lysozyme mutants and allow them to release some phage (see Abedon 1994). From the genetic data (Emrich 1968), it appears most likely that *sp* is encoded by one of the uncharacterized ORFs between genes 41 and 61. [Abedon (1994) discusses the compelling evidence against the suggestion made by

Obringer *et al.* 1988 that genes *40* and *sp* may be the same.]

Lysis inhibition requires a mechanism for determining the normal time of lysis, a signal for "phage excess," and a means of delaying lysis: Any model of lysis inhibition needs to deal with the following questions and observation:

How does the lysis clock operate in the absence of lysis inhibition? In trying to understand the regulation of the timing of lysis in T4, it is useful to consider the better understood lysis clock in bacteriophage λ (*cf.* Young 1992). In λ, the time of lysis is determined by the production of inhibitory (S107) and active (S105) forms of the holin protein, S (Blasi *et al.* 1990). Both forms are products of the same gene, with two nearby start sites giving an extra Met-Lys at the start of the inhibitory form. Lysis occurs when the ratio of the active form of S to the inhibitory form exceeds a certain level; thus, the clock becomes a reflection of the (unknown) means of translational regulation of the two forms. However, energy poisons can trigger early lysis even while the inhibitory form is predominant (Blasi *et al.* 1990). In the absence of a potential difference across the membrane, the inhibitory form also becomes an active holin. This mechanism results in propagation of holin activation once initiated, leading to rapid lysis of the cell.

T4 shares with λ the ability to trigger early lysis in response to energy poisons in a holin-dependent manner, independent of *r* gene function (Doermann 1952; Josslin 1971; Young 1992). It is conceivable that lysis by an energy poison reflects activation of an inactive form of gp*t* analogous to the inhibitory form of S. However, there is no evidence that the T4 *t* protein has two such counterbalancing forms.

Normal lysis in T4, as in λ and other phages, is precisely timed. We have no evidence as to the actual nature of the clock mechanism. There is potentially a strong stem-loop in the middle of the *t* gene that could possibly lead to a delay in completion of its translation until some other signal is received or to a truncated form of gp*t*. Furthermore, it appears that there may be more to the normal lysis clock than just allowing lysozyme access to the murein layer. Surprisingly, Fraser *et al.* (1957) report that T2-infected cells converted to spheroplasts after infection lyse at the same time as intact cells, although no murein layer is present and thus lysozyme is not involved.

What is the direct signal for "phage excess"? Several lines of evidence suggest that the lysis-inhibition signal must come from a stage later than the contact between the tail and the outer membrane or the penetration of the peptidoglycan layer:

1. Lysis inhibition of T4-infected cells is induced by all other T-even phages tested and by UV-inactivated phage but not by phage ghosts, whose heads have broken open through osmotic shock (Rutberg and Rutberg 1965). Ghosts can still attach to cells and penetrate the peptidoglycan layer, but they have no DNA or internal proteins to release into the periplasmic space.
2. LIN is not induced by superinfection of T4-infected cells with other unrelated phages including T7, which has a very different DNA ejection mechanism and would not be likely to release the DNA into the periplasmic space (Rutberg and Rutberg 1965).

These results make it appear likely that the DNA, the internal proteins IPI, IPII, and IPIII, internal peptides generated during cleavage of head proteins, and/or some other small DNA-binding molecule packaged in the head is responsible for signaling phage excess.

What is the receptor for the lysis-inhibition signal, and what response(s) does it mediate? On the basis of the work presented here, it appears very likely that the *rI* gene product is at least part of the receptor for the LIN signal. With the prediction (discussed above) that *rI* probably is secreted into the periplasmic space, the most logical suggestion is that it interacts directly with the signaling molecule there. We have looked for possible nucleic-acid- or nucleotide-binding motifs in the *rI* protein, but have not found any of the common ones, or any other suggestions as to what the specific mechanism of signal reception might be.

The triggering of the "external phage excess" signal has two obvious consequences. There is a major delay in the functional assembly or the opening of the holin pore that allows lysozyme to move out into the periplasmic space. Also, there is a delay in the loss of membrane potential and metabolic energy that occurs at the normal time of lysis, even in lysozyme mutants. For example, in t^+e^- infections, a single superinfecting phage extends respiration for just as long as the delay in lysis in a t^+e^+ infection under similar LIN conditions (Bode 1967; Mukai *et al.* 1967).

The ability to induce lysis inhibition as early as 3 min suggests that triggering the response to superinfecting phage can occur in the absence of gp*t*, which is a late protein. Both the effects of energy poisons and the observation that superinfecting phage can induce LIN after some cells have already begun to lyse are most compatible with post-translational regulation, although translational regulation can not be ruled out.

Is *rII* really involved in lysis inhibition? Several lines of evidence raise the interesting possibility that the *rII* genes may not actually play a role in lysis inhibition; rather, it appears likely that *rII* mutants simply trigger early lysis by an alternative pathway that is not subject to LIN. Evidence considered in building this alternative model includes the following:

1. While *rI* is required for lysis inhibition on all host strains tested, *rII* is not. Only on B do *rII* mutants make large, sharp-edged plaques; the *rII* genes are not required for LIN on K strains (Benzer 1957) or on the Bc variant of B (Rutberg and Rutberg 1964),

which differs from B in having lost a defective prophage related to P2.
2. On *E. coli* B strain S/6/5, all *rIIA* and *rIIB* mutants tested gradually lyse about 5 min *before* cells infected with wild-type T4, as shown above.
3. *rII* mutants partially suppress the constitutive lysis-inhibition phenotype of *t* mutants, thus suggesting that they are not operating in the same pathway.

Some of the other phenotypic effects of the *rII* mutations may in fact give us clues as to why these mutants are unable to establish lysis inhibition in response to superinfecting phage:

1. The *rII* gene products seem to play a role related to cell energetics, which is apparently required at least in cells lysogenic for λ. Infection is initiated normally in these lysogenic strains, but by ~12 min after infection, all energy-dependent processes come to an abrupt halt (Parma *et al.* 1992; Snyder and Kaufmann 1994). This includes gene expression, Mg^{2+} transport, and ATP biosynthesis.
2. The substitution of Mg^{2+} and other divalent cations for monovalent cations leads to substantial rescue of *rII* mutant infections of K-12(λ) (Garen 1961).

This inhibition of *rII* mutants during infection of λ lysogens depends on the λ *rexA* and *rexB* genes (Parma *et al.* 1992). It also affects particular mutants of phages T5 and T7, which do not lysis-inhibit. Their evidence suggests that the *rexB* protein is an ion channel-specific for monovalent cations, a channel that must be activated by gp*rexA*, with the two functioning as a two-component regulatory system at least one function of which is to sense phage infection. Overproducing gp*rexA* relative to gp*rexB* can lead to a loss of membrane potential and of energy production even in the absence of phage infection, and even wild-type T4 is restricted if both are overexpressed from a multi-copy plasmid (Parma *et al.* 1992).

We suggest that on the common B strains, just as on K-12(λ), the *rII* genes are required for maintaining membrane integrity and for sustaining energetics, but the membrane breakdown is slower and there is time for a substantial number of phage to be made before the cells break down. B is known to contain a cryptic prophage related to P2 which is required for the *rII* rapid-lysis phenotype (Rutberg and Rutberg 1964); curing it of that prophage leads to wild-type plaque morphology with *rII* mutants, while restoring that phage or P2 brings back *r*-type morphology. This phage may have a less effective *rex*-system analogue or some other mechanism of gradually destabilizing the membrane after infection, which can also be counteracted by the wild-type T4 *rII* genes. Lack of *rII* would then lead to a truncated infection, which triggers lysis, in the same way that energy poisons cause infected cells to lyse early. In this scheme, instead of *rII* mutations preventing the delay of lysis after superinfection, they simply allow infected cells to lyse in a mode unaffected by lysis inhibition, late enough that a significant burst of phage is still released.

P2 does have its own complex set of lysis genes, including not only a holin and an endolysin but two nonessential genes that seem to affect the timing of lysis (Ziermann *et al.* 1994). These are not expressed in lysogens, but it is conceivable that some portion might be activated by T4 infection in the absence of the *rII* protein to give the rapid-lysis phenotype. P2 also has at least four genes apparently acquired by horizontal transfer which affect the growth of other phages (Calendar *et al.* 1998); at least one of those is membrane-related. Strain-specific host differences are also important. For example, K-12 lysogenized with P2 gives wild-type plaques with *rII* mutants, showing neither the altered plaque morphology P2 induces on B nor the lethality *rII* mutants experience on K-12 λ lysogens.

This ability to generate an alternative way out of the cell, at least on some host strains, would explain why *rII* mutants can partially suppress the lysis defect in *t* mutants. If this model is correct, the *rII* genes (and the still uncharacterized *rex*-sensitivity genes of T5 and T7) give yet another example of the sophistication of the ongoing competition between bacteria and lytic phages, where the phage develop new modes of attack and the bacteria find ways to subvert them. A good deal of experimental work is clearly still needed to test this interpretation.

It would indeed be ironic if the rapid-lysis phenotype of *rII* mutants is at best tangentially related to lysis inhibition and primarily reflects a very fortuitous choice by Max Delbrück of the primary host strain to be used for phage studies. It seems to mean that both of the key phenotypes of *rII* mutants so crucial for those early genetic studies—the large-plaque morphology on B, permitting easy identification of mutants, and the lethality on λ lysogens, permitting selection of revertants—are in fact related to the same basic cellular functions of *rII*. It is to be hoped that this new way of thinking may facilitate the sorting out of the mechanisms of lysis inhibition and of the *rII* effects, independent of each other. However, we still have no specific clues beyond possible *rII*-protein sequestration of host ligase to explain those phenotypes of *rII* mutants related to DNA replication, recombination, and packaging discussed in the introduction, such as suppression of the lethality of DNA ligase and some gene 32 mutants and potentiation of gene 49 mutant effects.

***rIII* and lysis inhibition:** The arguments related to *rII* leave open the question of whether or not *rIII* is directly involved in LIN, at least on some strains. On some K strains, it reportedly is not required for LIN (Benzer 1957). However, its complex pattern of gene regulation is virtually identical to that for *rI* and, unlike *rII* mutants, neither *rIII* nor *rI* mutants can rescue *t* mutants (Josslin 1971). No conditions have been found in which the absence of *rIII* is lethal, so we have no clues to its func-

tion as we do with the *rII* effects on λ lysogens. Relatively little physiological work has yet been done with *rIII* mutants; clearly, that is needed before further conclusions can be drawn.

The evolutionary significance of lysis inhibition has been explored in some detail (Hershey 1946; Doermann 1948; Abedon 1989, 1990, 1994; Young 1992). The rather short latent period allows phage to quickly overtake virgin populations of host cells; then, when uninfected cells become scarce, LIN allows for increased progeny production and prevents progeny from uselessly adsorbing to infected cells. The lack of lysis inhibition when *E. coli* B is infected with an *amber* gene 37 mutant at high cell density confirms that the progeny phage released from the first few lysing cells trigger the signal for external phage excess, and that there is not some other signal of dense cell conditions that triggers lysis inhibition. The conservation of *rI* in T-even phage is not surprising, given that *rI* is the only *r* gene definitely required for lysis inhibition on all host strains tested.

The results on apparent conservation of *rI* and *rIII* for RB69 are of particular interest because RB69 appears not to sport any traditional large-plaque, *r*-type mutants, despite very extensive screening (Smith and Drake 1998), and there had been much speculation that RB69 might not be capable of lysis inhibition. However, we find that RB69-infected cells do indeed undergo lysis inhibition in liquid culture at standard cell densities; when growing stocks, these cultures generally need to be lysed with chloroform after several hours, as is true for r^+T4 stocks.

In summary, the rapid-lysis phenotype seems to have at least two possible causes. It can reflect a direct block along the pathway leading to the lysis inhibition which results from superinfection; the evidence indicates that *rI* plays a key role in this pathway. In addition, rapid lysis can result from factors interfering with membrane integrity and energetics. Energy poisons late in the infection cycle initiate lysis in some way that still depends on *t* but cannot be prevented by superinfecting phage, even when that superinfection (and thus the triggering of lysis inhibition) occurred long before the energy poison. We propose here that *rII* mutants act via this second pathway, leading to truncated infections on B as they do on λ lysogens, with the presence and size of the plaque reflecting the exact time at which this breakdown of membrane integrity occurs. It is not yet clear which of these two mechanisms is involved in the *rIII* rapid-lysis phenotype. While we cannot yet make a precise model either for the normal lysis clock or the method of induction of lysis inhibition, the parameters are much more clear, and testable predictions can be made.

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