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Interactions in Lambdoid Bacteriophage Populations

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INTERACTIONS IN LAMBDOID BACTERIOPHAGE POPULATIONS

(TITLE)

BY

Bruce Edward Mitchell

THESIS

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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IN THE GRADUATE SCHOOL, EASTERN ILLINOIS UNIVERSITY
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INTERACTIONS IN LAMBDOID BACTERIOPHAGE POPULATIONS

BY

BRUCE EDWARD MITCHELL

B. S. in Zoology, Eastern Illinois University, 1975

ABSTRACT OF A THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Zoology at the Graduate School
of Eastern Illinois University

CHARLESTON, ILLINOIS
1975

The lambdoid bacteriophages are ideal subjects for the study of populational interactions at the molecular level because they do not mutually exclude one another in mixed infection of the same host cell. Previous work done in this laboratory using the lambdoid phage pairs λ-434 *hy mi* and λ-Ø80 have demonstrated a density-dependent reduction in progeny yield per infected cell (burst size) as a function of increasing multiplicity of infection (m.o.i.). The λ *cI* gene which codes for the repressor molecule has been demonstrated to be the cause of the interaction in the λ-434 *hy mi* phage pair.

The purpose of this research was to determine if the λ *cI* gene is also responsible for the interaction observed in the λ-Ø80 phage pair. The λ *cII* gene which also functions in an immunity specific manner was also considered as a source of interaction. The experiments were conducted using the mutant phages λ*cI*$_{857}$ and 434 *hy cII*68, a hybrid phage consisting of the 434 immunity region in an otherwise λ genome. By comparing the lines generated on a log-log plot of burst size versus m.o.i. it is possible to determine whether these phages deficient in the λ *cI* or *cII* genes produced results similar to the interaction

observed in the wild type phage pairs. If the interaction is seen to be eliminated by the use of a mutant phage, then the interaction is dependent upon the gene that is deficient in that mutant phage.

The results of this study indicate that the interaction observed in the λ-Ø80 phage pair is not dependent upon the λ cI or cII genes. Neither mutant phage alleviated the density-dependent decrease in burst size. This suggests that some vegetative gene is responsible for the observed interaction. It is postulated that the most likely candidates are the DNA replication genes (O and P) and the gene (Q) which controls late phage transcriptions.

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INTRODUCTION

Evolution at the molecular level and the application of the principles of natural selection to molecules have been discussed by Lewontin (1970). He suggested that selection at the molecular level should be observable in a population of mixed, non-excluding phage types that have multiply infected a bacterial host cell.

Research done using an *in vitro* replicating system for phage $Q\beta$ RNA has shown that self-replicating variants evolve rapidly under intense selection for high replication rate or utilization of media deficient in necessary materials (Mills, Peterson and Spiegelman, 1967 and Levisohn and Spiegelman, 1969). It has also been demonstrated that the structural "phenotype" which is determined by secondary and tertiary conformations, is the object of selection (Mills, Kramer and Spiegelman, 1973).

Another case of selection being applied at the molecular level was observed in a study by Campbell, Lengyel and Lanridge (1973). Under intense selection for lactose utilization, an *Escherichia coli* strain deficient in β-galactosidase progressively evolved a new gene for a protein with complete β-galactosidase activity. This may be the process by which bacterial genes with new

functions involved.

Because the lambdoid bacteriophages do not exclude each other in mixed infection, they would seem to be ideal subjects for the study of molecular interactions at the populational level. Baumgardner, Elseth and Simmons (in preparation) have used this idea to do a series of studies on various lambdoid phages in mixed infection. By using the phage pairs λ-Ø80 and λ-434 hy mi they have demonstrated that there is indeed a populational interaction at the molecular level. It was also determined that the interaction, termed interference, was caused by the λ cI gene in the latter pair.

The purpose of this paper is to complete a part of the aforementioned studies. As the cI gene was determinate of the interference effect observed in the λ-434 hy mi phage pair, and because these two phages are very similar, it would be of interest to learn if the cI gene also caused the interaction effect observed in the λ-Ø80 phage pair. On the basis of nucleotide sequence homology Ø80 is the most distant relative of λ among the lambdoid phages (Simon, Davis and Davidson, 1971). The purpose of these experiments is to determine if the immunity specific genes, cI and cII, are responsible for the interaction between λ and Ø80 populations.

REVIEW OF LITERATURE

THE LAMBDOID PHAGES

The lambdoid bacteriophages are variants of naturally occurring temperate coliphages. The primary host for this group of viruses is the common intestinal tract bacterium, Escherichia coli. Being temperate indicates that the phages display differing modes of propagation dependent upon the molecular environment of the phage DNA.

The DNA of all phages in this group have three common characteristics: (1) UV inducibility, (2) possession of identical pairs of cohesive ends, and (3) recombination when intercrossed (Hershey and Dove, 1971 and Murray and Murray, 1973). The lambdoid phages are distinct from a second group of temperate phages, including P2, which hold none of the characteristics enumerated above (Bertani and Bertani, 1971).

Phages in the lambdoid group include those with immunity regions of λ, Ø80, 434, 21, Ø81, 82, and 424. There is little correlation between the immunity type and other characteristics exhibited by these phages (Hershey and Dove, 1971).

Heteroduplex DNA molecules are formed by hybridization of one strand from one type and the complementary

strand from the other type of phage. By using this process it is possible to do electron micrographic mapping studies which identify regions of homology and non-homology between the two strands. From these maps it is also possible to determine the percent homology of nucleotide base pairs between the two phage types. These studies revealed that lambdoid genome pairs range from 25 - 62% homology. It is also observed that segments of the two strands are either identical or altogether different. Segments which are homologous lie approximately equidistant from the left end of their respective DNA molecules (Westmoreland, Szybalski and Ris, 1969 and Fiandt et al, 1971). These results provide physical evidence that the lambdoid phages have derived from a common genome, and that while some regions of phage DNA have remained remarkably unchanged, others have diverged widely (Hershey and Dove, 1971). The evolutionary aspects of this apparent DNA segmentation will be discussed below.

Lambda and Ø80 are distinguished by differing immunity regions which determine the repressor molecule, among other proteins. Prophage location in the E. coli chromosome also differs as lambda inserts near the gal genes, and Ø80 near the trp operon. (Franklin and Dove, 1969). A study of nucleotide sequences of λ and Ø80 reveals that there is about 25% homology (Fig. 1). Ø80

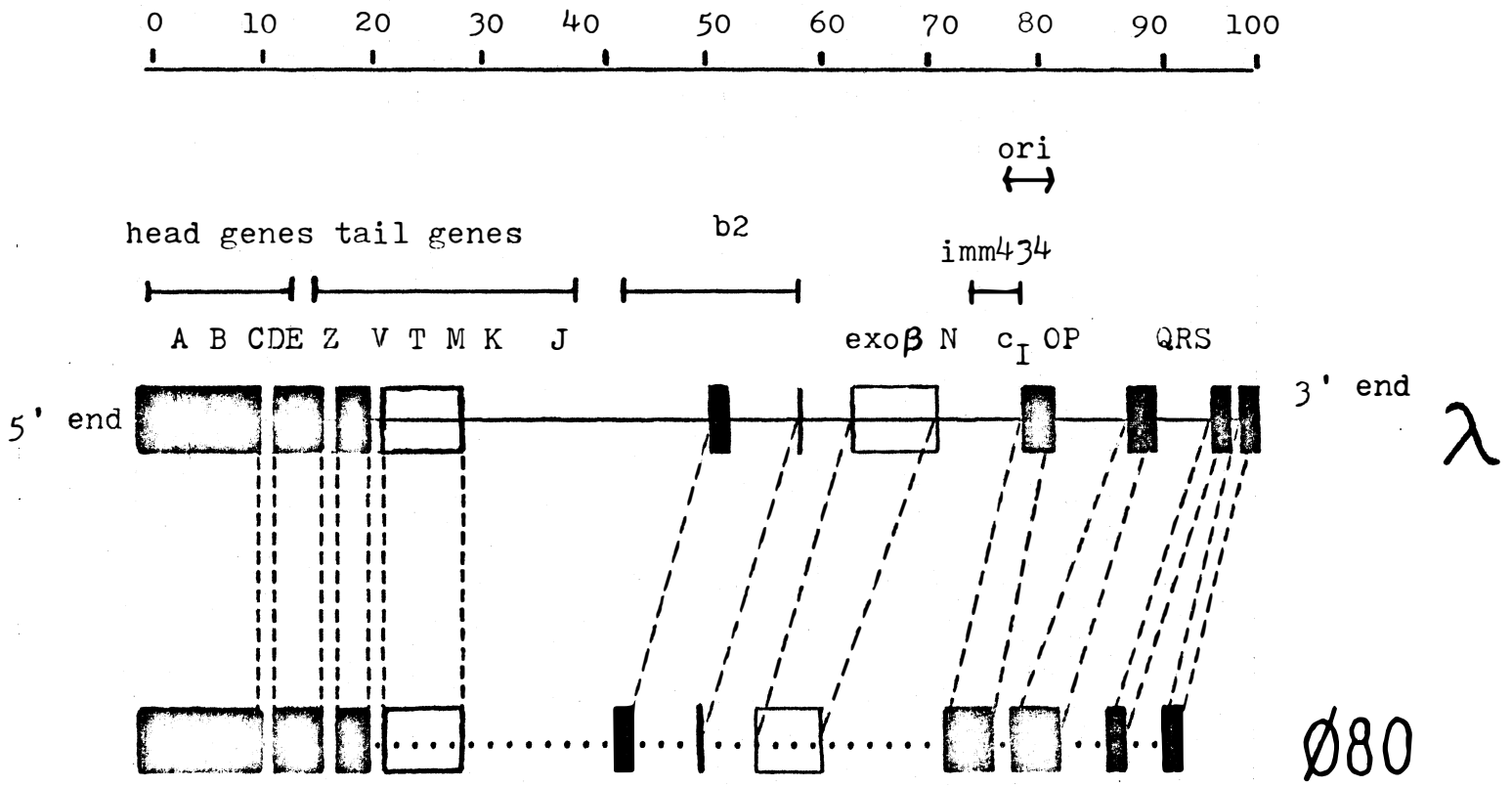


Figure 1. Genetic and physical maps of λ and Ø80 bacteriophages. All dimensions are in units of λ DNA length taken as 100 (upper scale). The genomes of λ and Ø80 are represented as solid and dotted lines respectively. Regions of strong homology between the nucleotide sequences of the two phages are represented as shaded rectangles and regions of variable homology as open rectangles. The relative positions of genes on the Ø80 map are identical to those given for λ. The line marked imm434 delineates the λ DNA segment that is lost by substitution in λ 434 hy. This figure is reproduced from Fiandt et al., 1971.

is then shown to be the most distant relative of λ among the lambdoid phages on the basis of nucleotide sequence homology. Their known biology, molecular structures and genetic maps have been shown to be quite similar (Simon, Davis, and Davidson, 1971).

PHYSICAL PROPERTIES

Phage particles

Studies using the electron microscope have elucidated many of the physical characteristics of mature λ and Ø80 particles. A curved, cylindrical tail 150 nm long is bound to a hexagonal head 54 nm in diameter (Kellenberger and Edgar, 1971). The head consists of 300 - 600 protomers which are hexons and pentons. The tail is seen in a stacked disc configuration with no plates, collars, or filaments being observed. The molecular weight for lambda particles has been determined as 5.7×10^7 daltons using equilibrium sedimentation (Dyson and van Holde, 1967). Work done with Ø80 particles fails to demonstrate significant differences with lambda particles (Shinagawa et al, 1966). This is to be expected since lambda heads have been shown to attach Ø80 tails (Weigle, 1968).

Phage DNA

The mass of lambda DNA has been calculated by

two different methods. Electron microscopy has revealed a double stranded, 17 nm linear molecule. Using comparison with phage T7 DNA, a molecular weight of 3.0×10^7 daltons was predicted (Davis and Hymen as cited by Kellenberger and Edgar, 1971). This molecular weight was confirmed by sedimentation velocity studies (Friefelder, 1970).

The mass of Ø80 DNA was determined by sedimentation velocity centrifugation is 2.93×10^7 daltons (Yamagishi, Yoshizabo, and Sato, 1966) which corresonds well with a molecular length equal to 92% of the lambda genome (Fiandt et al, 1971).

Mature lambda DNA is a linear, double-stranded helix with 5'-hydroxyl-terminated single strands extended from both ends (Strack and Kaiser, 1965). These single strands complement each other and thus allow the formation of closed monomers, open dimers, and open trimers in vitro (Hershey, Burgi, and Ingraham, 1963). Wu and Taylor (1971) elucidated the length and structure of the cohesive ends by sequencing. The 12 nucleotides on one strand are perfectly complementary to those on the other.

The exact nature of how the mature λ DNA is arranged within the phage head is as yet unknown. One study which caused ejection of the DNA revealed round, protein cores inside the heads of 35% of the phage coats.

It was postulated that perhaps lambda DNA is wound about these cores while contained in the phage head (Kaiser, 1966).

DNA segmentation

The DNA molecules of λ and Ø80 are divided into halves with respect to base-pair composition. As can be seen in Fig. 1, the left halves of their respective genomes exhibit extensive homology whereas the right halves differ considerably. This is reflected by base-pair composition, as λ DNA varies from 57 to 37% GC content from the 5' to 3' end, whereas Ø80 DNA varies from 55 to 50% GC content. Considering this large difference in GC content in light of the fact that λ and Ø80 have a similar organization of functional and control mechanisms, Skalka (1969) proposed that base-pair composition may not be a significant determinant of gene product activity.

The DNA molecules of λ and Ø80 can be fragmented by use of hydrodynamic shear techniques. Once fragmented a density fractionation technique employing the preferential binding of mercuric ion with AT rich segments can be used to separate the fragments according to their base-pair compositions. Six segments of varying base compositions were detected using this technique. These sharply delineated regions may be clusters of genes with similar functions (Skalka, Burgi and Hershey, 1968).

Many temperate phage DNAs including λ and Ø80

exhibit a good deal of intramolecular heterogeneity. In the case of λ it is most extreme as its DNA is clearly segmented. Ø80 is less so, but still exhibits segmented DNA. Arguments can be made that the segmentation represents the clustering of genes of similar function (Falkow and Cowie, 1968).

GROWTH CYCLE

The life cycle of a lambdoid virus begins with phage attachment to the E. coli host cell. This process is a function of tail protein and phage specific receptors on the bacterial cell surface. Using a buffered maltose medium it has been shown, by a study of the kinetics of phage adsorption, that there are approximately 6000 receptors on the cell surface (Schwartz, 1975). The attachment is reversible and can take place at 0^{o} C. The injection of the phage DNA into the cell requires ATP (Bode and Kaiser, 1965). The exact mechanism of injection is as yet not understood. Following penetration into the cell, the phage DNA has been observed to exhibit three different pathways for replication.

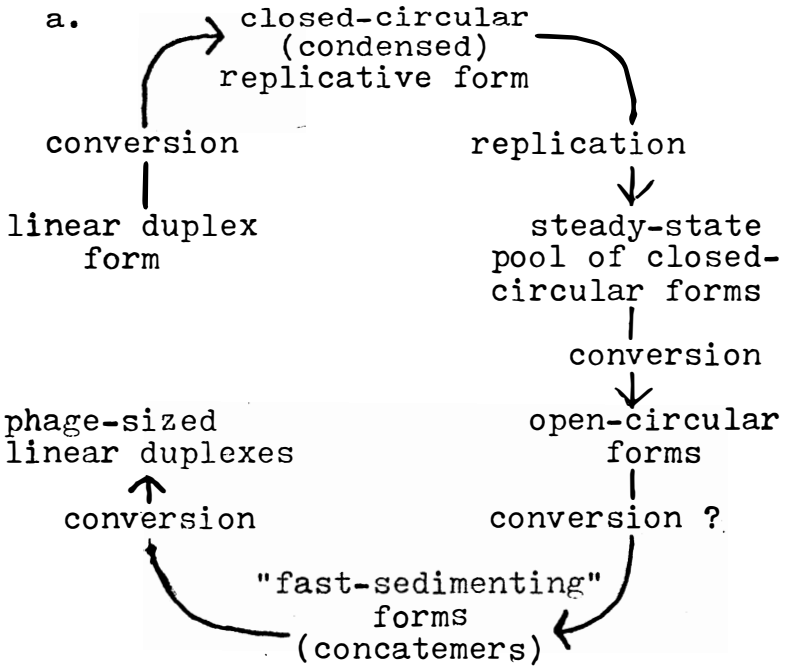
Lytic cycle

The most prevalent of these responses is known as the lytic, or vegetative cycle in which the phage DNA replicates independently of the bacterial chromosome, phage

protein synthesis occurs, mature viral particles are produced and then released by lysis of the host cell. Normally 99.9% of phage DNA's initiate the lytic cycle, which is described below and is illustrated by Fig. 2.

Within five minutes of DNA injection one finds the cohesive ends have joined, forming the covalently closed replicative conformation. Open circular forms are observed 9-15 minutes following infection (Kiger, Young, and Sinsheimer, 1968). In a casamino acids medium at 37° C semi-conservative replication of DNA is observed. A steady state pool of 20 closed circular molecules is maintained throughout the latent period as a template for production of a third "fast-sedimenting" type of DNA which first appears at 15 minutes (Carter and Smith, 1970). Denaturation of the latter molecule reveals long single strands indicative of concatameric molecules of lambda DNA. Pulse labelling experiments show that these concatamers are the precursors of mature lambda DNA (Carter, Smith and Shaw, 1969 and Salzman and Weissbach, 1967). The total DNA pool increases exponentially throughout the latent period. By 27 minutes phage-sized DNA has accrued at a rate parallel to DNA synthesis such that there are 90 phage DNA equivalents present (Kellenberger, 1961).

Lambda DNA replication appears to be similar to that of its host, E. coli. Electron microscopic studies



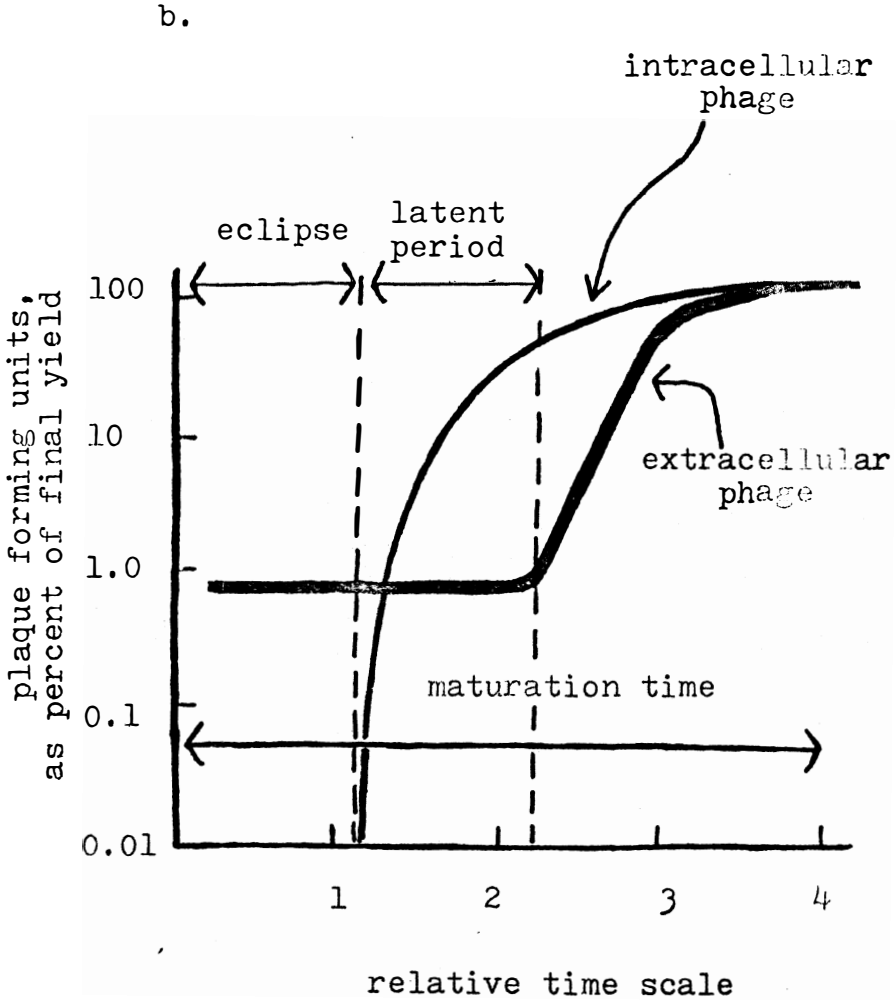


Figure 2. General description of the phage life cycle. (a)- diagram of the scheme of DNA replication; (b)- phage growth kinetics. (Taken from G. S. Stent, Molecular Genetics. Freeman, San Francisco, 1971)

by Schnös and Inman (1970) reveal a characteristic theta-shaped structure for replicating λ DNA circle. Lambda DNA replication is seen to originate at a unique *ori* site (Fig. 3) and progress bidirectionally. The requirements for λ DNA replication are the host replication system, the λ products of genes *O* and *P*, and the activation of *ori* transcription (Szybalski, 1971). The absolute amounts and times differ dependent upon the phage species and environment, but the preceding general scheme describes both λ and ø80.

Lambda DNA maturation occurs when the concatameric, "fast-sedimenting" form is split by site-specific endonucleases into phage-sized pieces. The DNA always enters the preformed spherical proheads 5' end first and 3' end last. The DNA is complexed with two proteins for condensation. Phage assembly requires certain undefined host (*E. coli*) products, as well as ATP in at least two steps (Becker, Murialdo and Gold, 1977). Phage assembly requires phage coded proteins as well. Mature phage are produced at a constant rate for 40 - 80 minutes. When intracellular lysozyme (*R* protein) concentration becomes high enough it will lyse the cell wall and release the mature phage particles (Jacob and Fuerst, 1958). After the DNA is incorporated into the prohead, the tails are attached. The mechanism of head-tail attachment for ø80

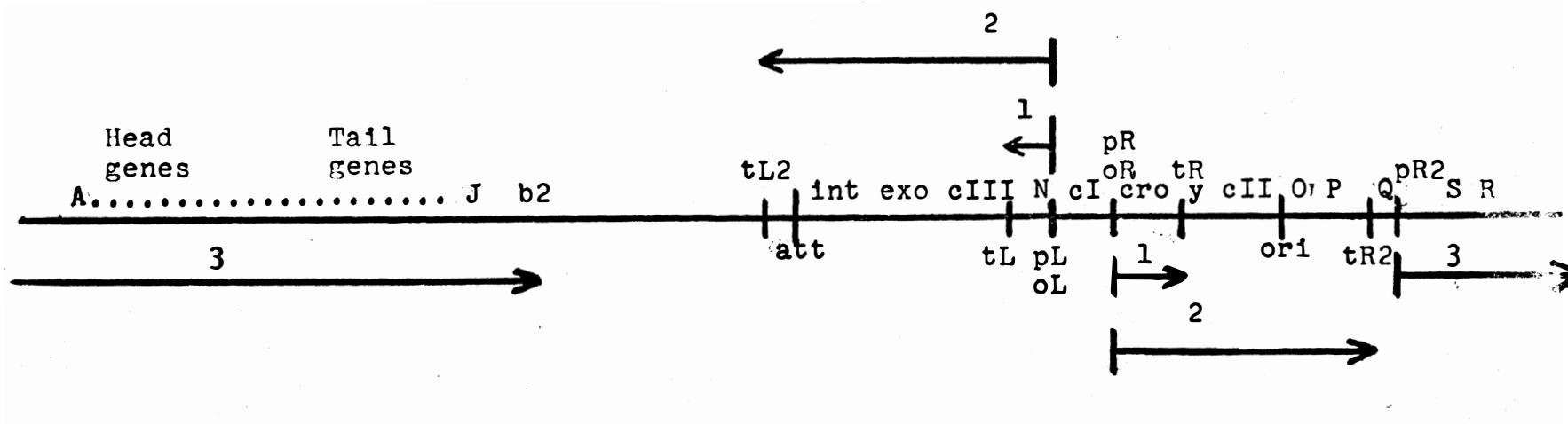


Figure 3. λ genetic map. Arrows indicate direction of lytic transcription. 1,2 and 3 correspond to the three sets of transcription described in the text. Promoters (pL, pR and pR2), terminators (tL,tL2, tR and tR2) and operators (oL and oR) are indicated.

is shown to be similar to that of λ by the fact that Ø80 tails attach to λ heads (Weigle, 1968).

The second type of response exhibited by lambdoid phages only applies to certain mutants of λ. Given these and special host conditions the DNA can be carried as an unstable plasmid. There is no coordination between host and phage DNA replication (Leib, 1970).

Lysogeny

In the third pathway phage DNA becomes integrated into the bacterial chromosome at a frequency of approximately 10^{-5}. The gene product of *int* is required. The viral DNA replicates at the same rate as that of the host cell. When integrated into the host chromosome the viral DNA is known as a prophage, and the cell is said to be lysogenic for the particular prophage. The λ prophage is located near the *gal* genes of *E. coli* and Ø80 is located near the *trp* genes (Kaiser and Hogness, 1960 and Matsushiro, 1963).

The lysogenic state is maintained through the activity of the *cI* gene product, the λ repressor. Repressor is found to bind operator regions adjacent to the promoters of early gene transcription. This prevents transcription of everything but the *cI* gene (Ptashne, 1971 and Reichardt, 1975a). The repressor has been shown to stimulate its own transcription and thus maintain the lysogenic state (Reichardt, 1975b). Induction of the

lambdoid prophage is the result of a stimulus which causes reduction in repressor activity and excision by a site specific endonuclease (*xis* gene product). Induction produces normal vegetative phage in a very high proportion of cases (Gottesman and Weisberg, 1971). Sometimes, however, a portion of the *E. coli* genome is excised in place of λ DNA resulting in defective phages. These defective phage have been extremely valuable in bacterial gene mapping (Morse, Lederberg and Lederberg, 1956).

REGULATION OF GENE EXPRESSION

Lytic cycle

Gene expression in λ is a well defined sequence of events (Campbell, 1971, Echols, 1972 and Herskowitz, 1973) involving early, intermediate and late controls. This sequence of events can be viewed fairly simply by first examining a genetic map of the λ chromosome (Fig. 3). The left and right halves are distinguished from one another in function as well as in nucleotide composition. The left half consists of the codons for the structural proteins (*A*-*J*) of the phage particle. The right half codes for the regulatory proteins which order events in the lytic, or vegetative pathway, and determine immunity. The center region consists of the genes which specify

recombination proteins.

Lambda DNA is large enough to code for approximately 50 proteins of molecular weight 33,000 daltons, and there have been about 50 λ proteins identified. Essential genes are defined as those necessary to form plaques on *E. coli*; 28 essential genes have been identified. These include genes *A* through *J*, *N*, *cro*, *O*, *P*, *Q*, *S* and *R*. The *cI*, *cII*, and *cIII* genes are considered non-essential (Hendrix, 1971).

The lytic cycle can be divided into three distinct sets of transcription. Upon infection of a host cell, the first set of transcriptions begins almost immediately. This early transcription requires no phage products, but does require host RNA polymerase. These early mRNAs are transcribed from the *N* and *cro* genes, beginning at the left (pL) and right (pR) promoters and ending at the left (tL) and right (tR) terminators respectively. The transcriptions are in opposite directions and therefore on different strands as RNA synthesis only occurs in a 5' to 3' direction.

The next set of transcriptions require the *N* gene product. These transcriptions also originate at the left and right promoters. Genes *cIII*, *exo* and *int* are transcribed leftward from pL, while *cII*, *O*, *P*, and *Q* are transcribed rightward from pR. The *N* protein is thought

to modify the host RNA polymerase in such a manner as to create a "juggernaut" polymerase which overrides tL and tR thus allowing the second set of transcriptions. A second set of terminators has been proposed. The tL2 site would lie just to the left of int, and tR2 would lie in the region of Q (Oppenheim, Katzir and Oppenheim, 1977).

After several minutes this second set of transcriptions is halted by the action of the cro product. Because this protein is active as a dimer, time is required for enough product to accumulate, thus facilitating the binding to the left (oL) and right (oR) operators. The cro product has only 1/100 the affinity for λ DNA as compared to the λ repressor (cI protein) (Takeda, Folkmanis and Echols, 1977).

The third set of transcriptions begins at about ten minutes after infection and includes all the late proteins (S, R and A through J) and requires the Q gene product. This mRNA is transcribed rightward from the late promotor (pR2) located in or to the right of gene Q. As the λ DNA is now circularized, transcription continues from gene R through the joined cohesive ends and then proceeds from gene A through gene J. Q protein may act as a RNA polymerase factor and thus allow transcription from this late promoter.

This temporal order alone does not elucidate the control system of λ. There is a delicate balance of gene products present from the first set of transcriptions. N product allows transcription of the genes necessary for DNA replication (O and P), late protein production (Q), recombination (exo, cII and cIII), and lysogeny (int, cII and cIII). The whole system can be viewed as a network of positive and negative feedback loops (Fig. 4).

Gene N product is the positive controller of the transciption of the intermediate regulators (O, P, Q, cII, cIII, and int). Intermediate control then is dependent upon the amount of N product originally produced. This system is balanced by the negative control of the cro dimer which halts transcription of these intermediate control genes. The lytic mode depends on cro as it not only halts the second set of transcriptions, but also that of N and itself. In addition, cro protein binds in the same area as the λ repressor, thus preventing repression and furthering the lytic cycle. cro is also thought to activate late gene transcription (Oppenheim et al, 1977).

The intermediate control system is also complex. The O and P genes required for DNA replication and as such have no direct controlling effect. However, if the O and P proteins stimulate DNA replication a pool of λ DNA is

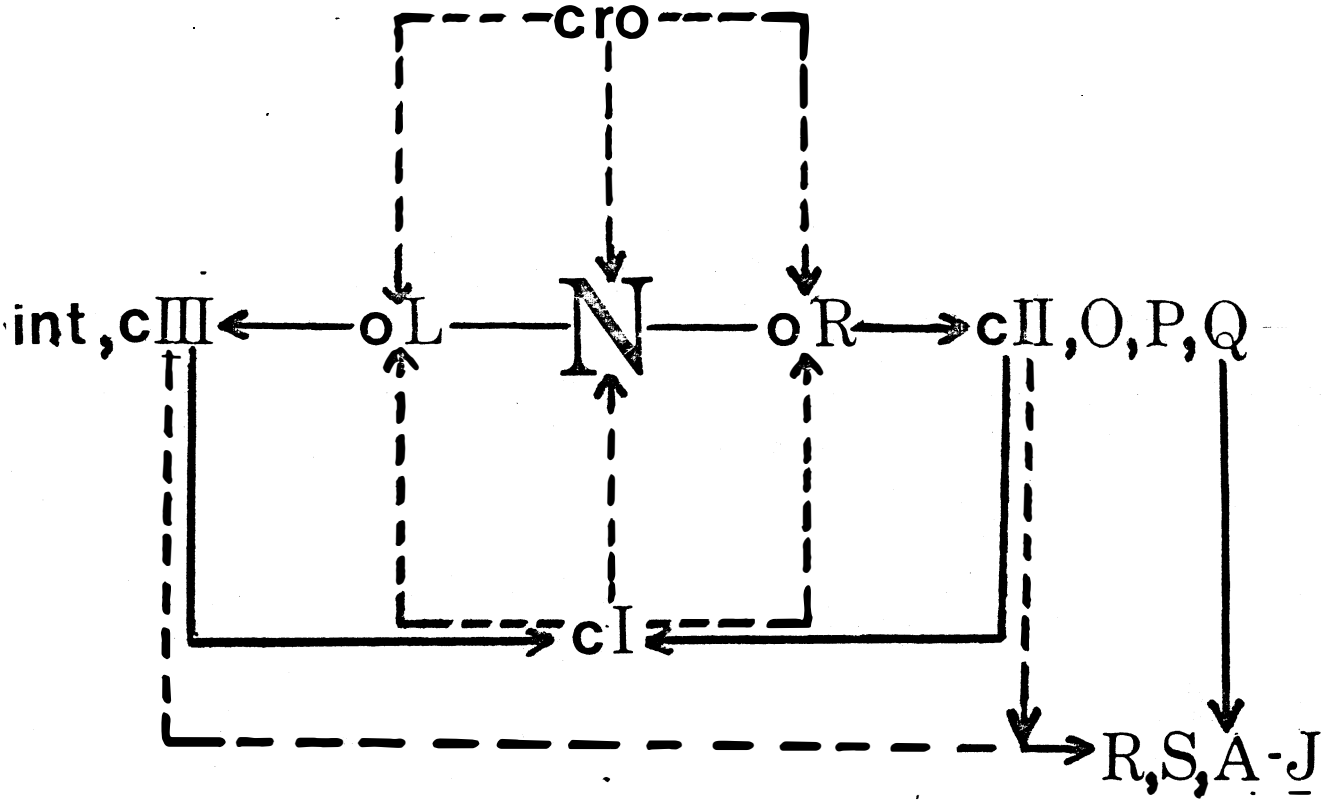


Figure 4. The λ gene regulation scheme. Dashed arrows indicate negative control; solid arrows indicate positive control. A dashed arrow can also be drawn between cro and cI and vice versa. Adapted from Herskowitz (1973).

accumulated. Transcription also takes place from these copies, so indirectly, O and P have an effect on what makes up the molecular environment of the λ chromosome.

The Q product is required for late protein production and acts as a positive control for late transcription.

The cII and cIII proteins are seen to perform at least a dual role in regulation. The cII/cIII products are positive controllers of int and cI mRNA synthesis. This is related to the establishment of lysogeny. These two gene products also function to negatively regulate late gene expression. The cII/cIII products act on a transcriptional level within the y region located to the left of cII, near tR, in both positive and negative regulation. The lytic cycle will continue unrepressed only if cro blocks transcription of cII and cIII before these concentration-dependent proteins can accumulate, and stimulate repressor transcription. If cro blocks transcription too soon, however, not enough O and P proteins are produced for replicative purposes (Court, Green, and Echols, 1975).

Lysogenic pathway

Establishment of lysogeny requires integration into the E. coli chromosome and repression of phage lytic transcriptions. The lysogenic response requires

int and cI proteins respectively. The transcription of these products is positively regulated by the cII/cIII gene products. int is the phage integrase enzyme responsible for integration into the E. coli chromosome near the gal gene locus at the attλ site on the phage chromosome.

The λ repressor molecule is coded by the cI gene. Transcription is initiated at the repressor establishment promoter (pRE) (Fig. 5). The site requires cII and/or cIII product bound at the y region. The direction of transcription is leftward and includes the cro gene in the anti-sense mode (3' to 5'). 5 to 27% of repressor mRNA is transcribed from pRE (Reichardt, 1975a).

The second cI transcription appears late (60 minutes) in the growth cycle and therefore does not affect the lysis-lysogeny decision. Transcription of cI now begins at the repressor maintenance promoter (pRM) and proceeds leftward. cI product is shown to stimulate its own transcription by binding to oR. This is the method by which cI represses prophage transcriptions. By binding oL and oR, repressor does not allow transcription of N or cro thus eliminating any possibility of lytic activity. At the same time it promotes its own transcription, thus preserving lysogeny. The cI

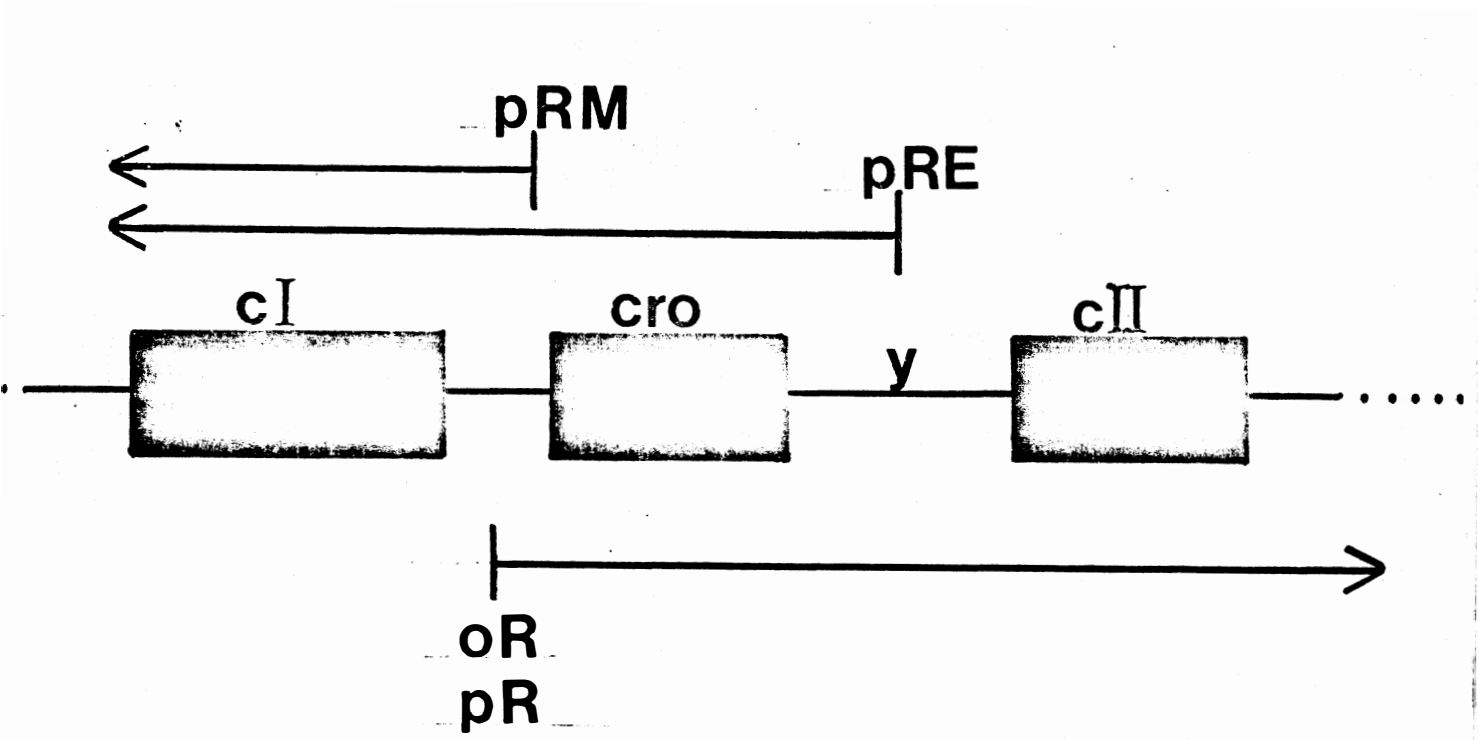


Figure 5. cI promoters and overlapping transcription. pRE- establishment promoter, pRM- maintenance promoter, oR- right operater, pR- right promoter. Arrows indicate direction of transcription (Herskowitz, 1973).

product is a negative regulator of all other genes and a positive regulator of itself.

Whether a phage follows the lytic or lysogenic pathway is dependent upon a delicate balance of cro, cII/cIII and cI products. The cII/cIII products are required for cI transcription and are thus positive regulators. The cro product has a dual role in the pathway decision. It inhibits cII/cIII transcription by binding near pL and pR and in this way controls cI production. As cro binds near pR which is located very near oR, it inhibits transcription from pRM. The cro and cI proteins have been shown to act at overlapping, but non-identical sites (Reichardt, 1975b). The cI protein has an affinity for λ DNA 100 times greater than the cro product. This indicates the possibility that the lysis-lysogeny decision is a function of the intracellular concentrations of cro and cI products which appear to be competing for the same site.(Takeda, Folkmanis and Echols, 1977). The lytic response may also be favored because transcription from one strand may be affected by that occurring on the complementary strand. Transcription of cro could be inhibiting cI transcription and thus favoring the lytic pathway.

The control of gene expression in λ is quite complex and features several proteins which are at least

bifunctional (cI, cII/cIII, and cro products). Much of the regulation is dependent upon molecular pool sizes (concentrations) and thus the O and P genes have an indirect effect by determining the number of transcribable DNA copies.

EVOLUTION

General Aspects

The lambdoid phages demonstrate that although base-pair composition may vary extensively, the genetic map is more readily conserved (Nahmias and Reanney, 1977). This may be a result of the clustering of genes of similar or related function to avoid the possibility of loss of vital DNA in the event of recombination. This arrangement of genes also allows for temporal coordination of the phage transcriptional control mechanisms. These clusters have been shown to be closely related to the segmented lambdoid DNA (Dove, 1971 and Murialdo and Siminovitch, 1972).

At present there are two hypotheses regarding the evolution of viruses. The first sees viruses as degenerative bacteria which have undergone a "retrogressive" evolution. The other has viruses originating from fragments of cellular polynucleotides which eventually become biologically active. There is evidence for

both arguments although much of it is ambiguous. The evolution of λ is quite complex and coupled with that of its host cell, E. coli (Nahmias and Reanney, 1977).

Molecular Aspects

Viral populations would seem to be ideal for the study of genetic interactions at the populational level. To date, however, very little work has been done in this area. The most significant work thus far has been done by Spiegelman's group using the RNA phage Qβ (Mills, Peterson and Spiegelman, 1967). Phage Qβ RNA can replicate in a mixture consisting of the replicase enzyme, RNA template, and RNA synthesis precursors. Using this in vitro system, and selecting for Qβ RNA molecules with a high replication rate, it was shown that RNA molecular evolution can take place. A 15-fold increase over normal in the RNA replication rate was observed. Three effects were noted: (1) an increased efficiency of complexing and reacting of the RNA with the replicase, which was possibly caused by (2) the elimination of 83% of the genome, and (3) a significant change in nucleotide composition. Studies conducted by Levisohn and Spiegelman (1969) using limiting concentrations of riboside triphosphates and base analogues demonstrated that a high degree of selection can occur between molecular variants when placed in a selective environment. Chain

lengths and base compositions were not changed. An allosteric effect of the RNA secondary and tertiary structure on the RNA replicase complex was proposed.

The complete sequence of Qβ RNA was reported in 1973 (Mills, Kramer, and Spiegelman). Sequencing revealed a molecule which contains oligonucleotids sequences which occur in both the plus and minus strands. This suggests that there is extensive base-pair bonding within the same strand which would give the RNA a characteristic secondary and tertiary structure. Selection could now be viewed in a new manner, resulting in a new distinction between genotype and phenotype in simple molecular species. The primary structure would be considered as genotype and the secondary and tertiary structure would determine phenotype; selection being exhibited as acting upon the "phenotype" of the molecule.

An analogous situation is observed in transfer RNA. Extensive base-pair bonding within a single-strand has been shown to determine the conformation of the tRNA secondary and tertiary structure and thus determines its activity (Rich and Kim, 1978).

In a study using β-galactosidase mutants of E. coli Campbell, Lengyel and Lanridge (1973) found that under intense selection for lactose metabolism a new gene for β-galactosidase was evolved. The gene maps at a

different place on the bacterial chromosome and there are differences in antigenic, enzymatic, and size properties between the evolved and normal types. The enzyme was produced by a series of mutations, possibly progressively changing a non-lactose gene into one capable of β-galactosidase activity. This may be the process by which new genes evolve through natural selection.

MIXED PHAGE INFECTION

Lewontin (1970) has suggested that selection should be observable on the molecular level when a host cell is multiply infected.

Early studies on mixed phage growth were largely conducted by Delbrück's group. Using the virulent T coliphages T4 and a mutant T4r Delbrück and Bailey (1946) found that two closely related phages could grow in the same host cell. Phage T4 will partially exclude phage T2 in mixed infections of a single host. Still more distantly related phages (T1 and T7, and T1 and T2) completely exclude one another (Weigle and Delbrück, 1951). The T phage studies seem to indicate that the greater the genetic disimilarity the higher the degree of exclusion observed. When a cell carrying the λ prophage is induced after prior infection by the phage T5, it is observed that mixed phage growth occurs in less than one percent of all cells, indicating mutual exclusion.

Mutual exclusion is not the only result expected between two related species. A study growing two strains of E. coli on a single resource has demonstrated that the exclusion principle is not always valid. Levin (1972) found that the two populations reach an equilibrium which is quite stable.

The only work to date studying lambdoid phage populations and their interactions is that done by Baumgardner, Elseth and Simmons (in preparation) of which this thesis is a part. These studies have demonstrated a density-dependent regulatory mechanism for lytic growth. As the multiplicity of infection (m.o.i.) is increased a line of negative slope is observed on a log-log plot of burst size versus m.o.i. This was found to be true of the phage pairs λ - 434 hy mi and λ - Ø80. The same effect was also noted with λ - λ and 434 hy mi - 434 hy mi pairs but was absent in the Ø80 - Ø80 pair.

As the phage-bacteria collisions are random, m.o.i. is defined as the mean (m) used in the Poisson distribution function

$$p(x) = \frac{e^{-m} m^{x}}{x!} .$$

An m.o.i. of three, for example would mean that an average of three phage attached to all bacterial cells. Placing the values in the Poisson function one finds that the probability of having an uninfected bacterial cell is

$$p(0) = \frac{e^{-3}\,3^{0}}{0!}$$

which is equivalent to

$$p(0) = e^{-3}.$$

This means about 96% of the cells will be infected by at least one phage at an m.o.i. of three. Burst size is the number of progeny phage produced per bacterial cell infected.

The negative slope was interpreted as a density-dependent reduction in progeny phage produced per infected cell as a function of increasing m.o.i. This was interpreted as meaning that there is a self-regulatory mechanism acting to guarantee an adequate host population for the phage. Such a system could operate through an increase in the probability of lysogeny at high m.o.i.

A striking similarity between the observed effects in single and mixed populations suggests that lambdoid phages are able to interfere with each others' lytic growth. These studies further determined that the interference effects were taking place during early phage functions and affecting the size of the phage DNA pools.

Using mutant phages to determine the gene(s) responsible for the interference effect, it was found that cII product was not the cause of the depression in phage yields in the λ-434 hy mi pair. 434 hy mi is a phage

possessing 99.3% of the λ genome, but with the 434 immunity region inserted in place that of λ's. The cI gene was found to produce the interference effect between the phages. As 434 hy mi and λ possess the same genome save for the immunity region, it was determined that the cI protein must have some function other than binding to the immunity specific left and right operators. It has been demonstrated that cI protein does positively regulate its own transcription (Reichardt, 1975b), and this could explain the m.o.i. effect on burst size of single phage populations. However, since the cI self-activation is immunity specific it cannot explain the density-dependent depression of phage yield observed between heteroimmune phages.

Media

Bacterial hosts were grown in K medium (0.075 M sodium-potassium phosphate buffer, pH 7.0, 0.02 M NH_4Cl, 0.005 M $MgSO_4$, 0.006 M NaCl and 1.5% casamino acids) supplemented with 2.4 mg/ml of maltose (KM medium). Phage-infected bacteria were grown in KG medium which is K medium supplemented with 2.4 mg/ml of glucose.

The nutrient broth (TB) contains 1.0% bacto-tryptone and 0.5% NaCl. Agar derivatives include TB soft agar (0.7% agar), TB plate agar (1.0% agar) and TB slant agar (2.0% agar).

Phage stocks were stored in TMB medium consisting of 0.01 M tris-HCl buffer, pH 7.5, 0.01 M $MgSO_4$ and 0.1 mg/ml bovine plasma albumin (BPA). All dilutions were made in TMB. During phage adsorption the bacteria were suspended in TM which is TMB with the BPA being deleted.

Bacterial Strains

Escherichia coli K12 W3104 (gal_4^-), a galactose-negative mutant of *E. coli* K12 deficient in galactose-1-phosphate uridyl transferase, was used in all interference experiments. W3104 was used as the indicator strain for determination of phage type by plaque morphology.

The appropriate lysogenic strains of W3104 were employed for differential plating used in the assays of phage titer. W3104 (gal_4^-) and its lysogenic derivatives were obtained from A. D. Kaiser and D. S. Hogness.

Bacteriophages

Phage Ø80 forms heterogeneously sized plaques that have halos and fuzzy edges when plated on W3104. The 434 hy cII_{68} mutant is a hybrid phage containing the immunity region of phage 434 in an otherwise λ genome which is mutant for the cII gene. The λ cI_{857} mutant contains a mutation in the gene which codes for the λ repressor molecule.

Phages Ø80, λcI_{857} and 434 hy cII_{68} were obtained from H. Echols and each was prepared by collection of the lysate from a liquid culture of infected bacteria. All phages were purified by a low-speed centrifugation (15 minutes at 5000rpm) to remove bacterial debris and a high-speed centrifugation (120 minutes at 15000 rpm) to remove the phage from the maltose medium, and resuspended in TMB. These separations were performed using a Sorvall Super-speed RC2-B centrifuge which has a centrifugal radius of 4.25 inches.

Maintenance of Bacteria

Bacteria were grown every three days from refrigerated single cell isolates stored on TB slant agar. These cultures provided innocula for bacterial hosts of phage growth and for the assay of phage titers. This method of maintaining W3104 guarantees a minimum of bacterial growth and therefore constrains genetic change in the bacterial population during the course of the experiments.

Interference Experiments

From the cultures grown every three days an innoculum of 1 ml was diluted thirty times in KM. The bacteria were grown to a density of 5×10^8 cells/ml as determined by precalibrated Bausch & Lomb Spectronic 20 absorbance (600 nm) reading. The bacteria were sedimented and resuspended in TM, concentrating two-fold. These bacteria were kept at 0° C until ready for use. For single strain experiments 1 ml samples of phage at concentrations corresponding to twice the desired m.o.i (multiplicity of infection) were added to 1 ml aliquots of bacteria, yielding a cell density of 5×10^8 per ml. Mixed phage infection was accomplished by adding 0.5 ml of the two phage types at concentrations corresponding to four times the desired m.o.i. to 1 ml aliquots of bacteria, also yielding

a cell density of 5 x 10^8 per ml. The samples were incubated at 37° C for 17 minutes and then diluted ten-fold into prewarmed (37° C) KGB. The phage-infected bacterial cultures were then incubated at 37° C for 120 minutes in a New Brunswick Gyrotory water bath. The cells were then lysed with 0.2 ml $CHCl_3$. Phage yields are expressed in burst size which is defined as the number of progeny phage (plaque forming units) produced per infected cell. As determined by the Poisson distribution function, at an m.o.i. of three or greater, at least 96% of the cells will be infected by at least one phage.

Phage Assays

To determine phage titers, 0.5 ml sample of the lysate from the interference experiments was taken through a sequential dilution series using ten-fold dilutions (0.5 ml sample into 4.5 ml 0° C TMB) until appropriate concentrations were obtained for plating.

Plating was conducted according to the soft agar overlay method. To 0.2 ml samples of assay bacteria were added 0.2 ml aliquots of the appropriately diluted phage samples. These were then incubated for 20 minutes at 37° C. The samples and assay bacteria were then mixed with 2 ml of TB soft agar and plated. The plates were

incubated overnight at 37° C.

By using the criteria of plaque morphology and immunity, the number of Ø80 plaques was determined. The number of plaques multiplied by the dilution factor gives the phage titer. The burst size is the quotient of the phage titer divided by the number of infected cells.

Results

To determine the effect produced by increasing m.o.i. of Ø80 on itself, experiments were conducted using a constant m.o.i. of 5. To this m.o.i. were added Ø80 m.o.i.'s of 3.5, 5, 7 and 10. Burst sizes were then determined. The results are tabulated in Table 1, and plotted in Figure 6. There does not appear to be a depressing effect upon Ø80 burst size with increasing m.o.i. in agreement with previous observations (Baumgardner, Elseth and Simmons, in preparation).

The effect produced by increasing m.o.i. of λ on Ø80 burst size was not measurable in this laboratory due to circumstances beyond the control of the experimenter. A determination of this effect has been made previously, and is seen in Figure 6 as a dotted line. The methodolgy used in this determination were identical to those used in this study excepting the phage pairs. (Baumgardner, Elseth and Simmons, in preparation). The use of this line for comparison seems justified as other results obtained in the course of these experiments were consistent with those obtained in the earlier work.

The burst size of Ø80 as affected by increasing m.o.i. of $\lambda \underline{cI}_{857}$ (hereafter designated $\lambda \underline{cI}$) was measured by using a constant m.o.i. of 5 for Ø80, while m.o.i.'s of 3.5, 5, 7 and 10 were employed in $\lambda \underline{cI}$ infection. The results are seen in Table 2 and are plotted in Figure 7.

Table 1. Effect of Ø80 on its own yields. (The Ø80 m.o.i. is 5.)

Added Ø80 m.o.i.	Ø80 burst size	Relative burst size
3.5	610	1.00
5	570	0.74
7	820	0.54
10	530	0.42

Table 2. Effect of cI on Ø80 yields. (The Ø80 m.o.i. is 5.)

	Ø80 burst size		
cI m.o.i.	exp. 1	exp. 2	Relative burst size
3.5	18.6	12.2	1.00
5	9.0	13.0	0.71
7	6.4	8.2	0.49
10	6.4	4.6	0.35

Table 3. Effect of 434 hy cII on Ø80 yields. (Ø80 m.o.i. is 5.)

	Ø80 burst size		
434 hy cII m.o.i.	exp. 1	exp. 2	Relative burst size
3.5	32.0	30.0	1.00
5	15.4	26.0	0.71
7	13.7	22.0	0.57
10	13.7	12.6	0.42

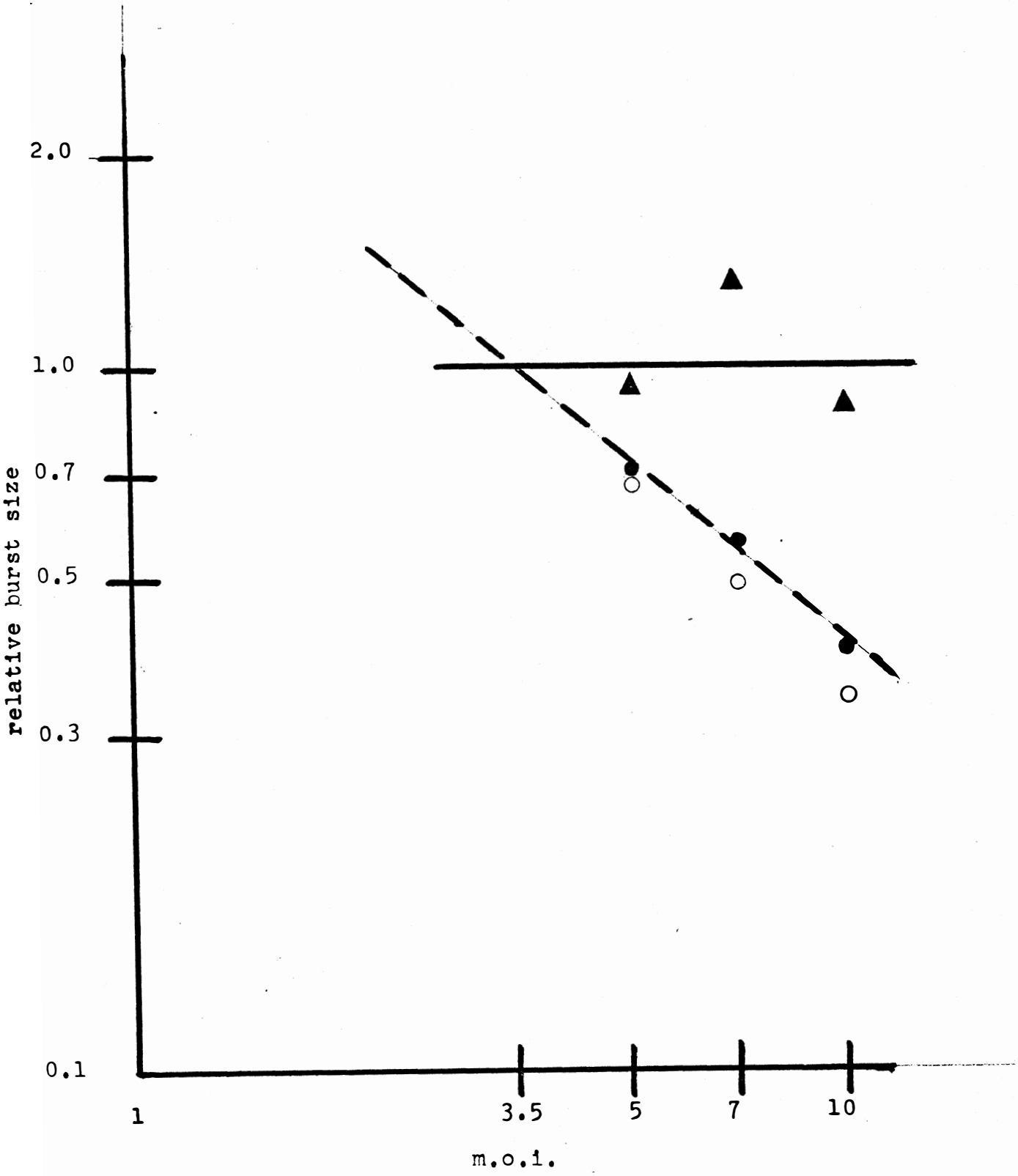


Figure 6. Burst size Ø80 (relative to m.o.i. 3.5) vs. m.o.i. of coinfecting phage. Dotted line is Ø80 vs. λ (see text for reference). Solid line is Ø80 vs. Ø80. ▲ Ø80, ○ λcI, ● 434 hy cII

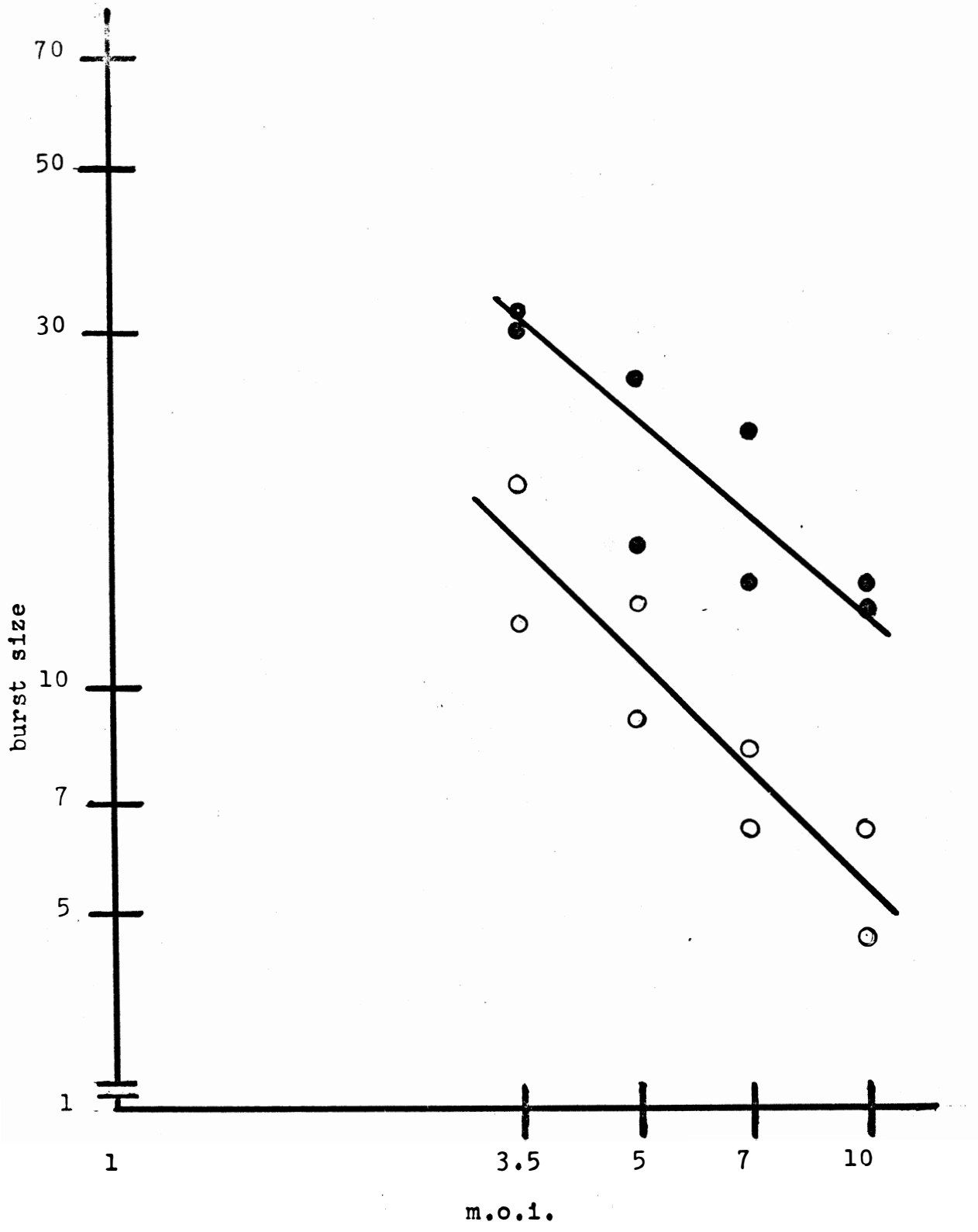


Figure 7. Burst size Ø80 vs. m.o.i. of coinfecting phage.
○ λcI (slope -1.00), ● 434 hy cII (slope -0.90)

It is observed that the cI gene is not responsible for the density-dependent depression of ø80 yields. This becomes quite apparent when one compares the plot of ø80 vs. λ with that of ø80 vs. λcI (Fig. 7) and observes that there is no significant difference.

The effect of the cII gene on ø80 burst size was measured by using a constant m.o.i. of 5 for ø80 and employing m.o.i.'s of 3.5, 5, 7 and 10 for 434 hy cII$_{68}$ (hereafter designated 434 hy cII). Phage 434 hy cII contains the phage 434 immunity region in an otherwise genome, so its cII gene is actually the λcII gene. The data are presented in Table 3 and are plotted in Figure 7. It is observed that the cII gene is not responsible for the interference effect of λ on ø80. This is apparent when the plot of ø80 vs. 434 hy cII is compared to that of ø80 vs. λ (Fig. 6). The difference in slopes does not appear to be significant.

The plotting of data on log-log coordinates was done to facilitate evaluation of the data by comparing the approximate slopes of the lines obtained. This method has been confirmed earlier in work done by Baumgardner, Elseth and Simmons (in preparation) of which this thesis is a part.

DISCUSSION

The results of this study demonstrate that the populational interaction observed between ø80 and λ is not caused by the cI or cII genes. The same density-dependent effect that was observed for λ is seen to hold for the mutant phages λcI and 434 hy cII. The approximate slope of the line for the ø80-λcI pair is -1.00, and that of the ø80-434 hy cII pair is -0.90. If the cI or cII gene was responsible for the interaction observed, one would expect that the slopes would be approximately 0 (the removal of the gene responsible for the interaction would make the ø80 burst size vs. m.o.i. graph a horizontal line as in the ø80-ø80 graph in Fig. 6). The fact that the points for the ø80-λcI and ø80-434 hy cII phage pairs fall very close to the line (slope -0.80) obtained earlier (Baumgardner, Elseth and Simmons, in preparation) (Fig. 6) for the ø80-λ phage pair is also supportive evidence for the conclusion that the cI and cII genes are not responsible for the effects observed. The two mutants generate lines of slightly greater slope than the λ wild type phage. This may be explained by the difference between the presence (wild type) or absence (cI or cII) of repressor molecule. Those types producing repressor will lysogenize at a certain frequency, thus eliminating any possibility of lytic interaction. Those lacking

normal yields (Herman et al., 1973). Comparison of Ø80 burst sizes from Table 1 with those in Table 2 and Table 3 reveal that Ø80 yields are reduced approximately 95 - 97%.

A comparison of the above results with the work done by Baumgardner, Elseth and Simmons (in preparation) using the λ-434 hy mi phage pair provides an interesting contrast. λ and 434 hy mi are observed to reduce each others' yields in mixed infections. This interaction was observed to be dependent upon λcI gene, but not the λcII gene. Phage 434 hy mi consists of the 434 immunity region in an otherwise λ genome, making λ and 434 hy mi very closely related. Considering this and the fact that Ø80 and λ are distantly related, these results lend support to the idea that exclusion, partial or mutual, is a function of genetic dissimilarity as postulated by Weigle and Delbruck (1951).

As recombination can occur between lambdoid phages, there is no guarantee that this and/or mutations have not affected the results obtained in these experiments.

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