Switches in Bacteriophage Lambda Development*

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Abstract

The lysis-lysogeny decision of bacteriophage lambda (λ) is a paradigm for developmental genetic networks. There are three key features, which characterize the network. First, after infection of the host bacterium, a decision between lytic or lysogenic development is made that is dependent upon environmental signals and the number of infecting phages per cell. Second, the lysogenic prophage state is very stable. Third, the prophage enters lytic development in response to DNA-damaging agents. The CI and Cro regulators define the lysogenic and lytic states, respectively, as a bistable genetic switch. Whereas CI maintains a stable lysogenic state, recent studies indicate that Cro sets the lytic course not by directly blocking CI expression but indirectly by lowering levels of CII which activates *cI transcription*. We discuss how a relatively simple phage like λ employs a complex genetic network in decision-making processes, providing a challenge for theoretical modeling.

Contents

For the concert of life no-one gets a program.

[From a Dutch tile (81)]

INTRODUCTION

A central challenge in the post genomic era is to understand processes governing the dynamics of highly complex genetic regulatory networks. A growing number of theoretical modeling investigators are attempting to explain the underlying principles of complex regulatory networks involved in normal mammalian development, including alterations that can result in a disease state such as cancer. Analysis of such complex systems would be greatly facilitated by similar studies using an ideal paradigm in which most if not all of the elements composing the system were known. Phage λ, the most comprehensively studied bacteriophage, is the prototype of a class of lambdoid phages with whom it shares similar genome organization and functions (38, 82). Studies of λ that began in the 1950s continue to reveal key molecular processes in gene regulatory mechanisms and development. However, despite years of study, many genetic interactions still remain to be uncovered and those that we already know require reexamination. For an accurate, complete, and quantitative analysis of the genetic network, in particular its temporal progression, these remaining questions need to be addressed. In this review we summarize a systems biology approach to the study of genetic regulatory circuits of phage λ. We define the individual components of the circuits and switches, describe the kinetics of their interactions, and explain how the interactions achieve robustness in the performance of the circuits. We also stress some puzzles that still exist in lambda's regulatory system.

Our citation of literature is not exhaustive but provides examples to illustrate specific points. We direct the reader to several comprehensive reviews on phage biology (13, 15, 24, 31, 34, 37, 41, 82, 107).

The λ System

Bacteria and their temperate phages, like *Escherichia coli* and λ, exist in symbiotic relationships. These phages can be present in a dormant, lysogenic (prophage) state replicating passively with the host or they can develop lytically, producing progeny phages and

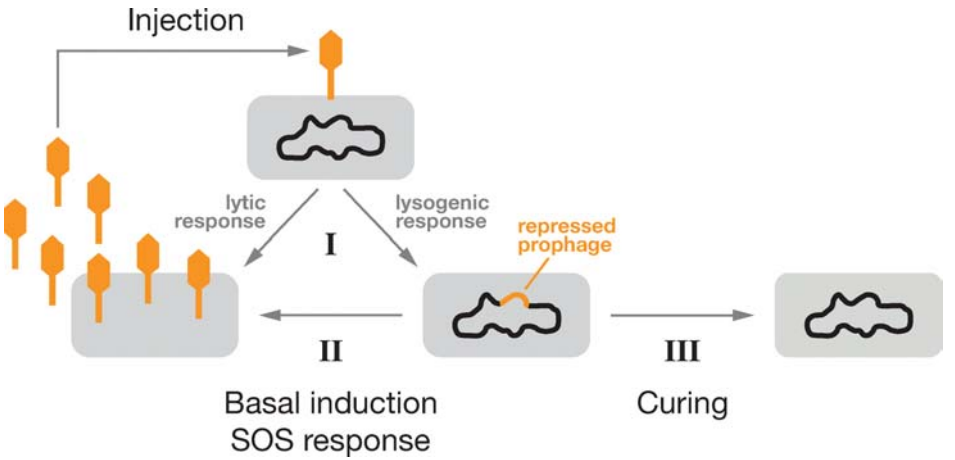

Figure 1

Decision-making steps by the temperate λ phage. A cell infected with phage λ can follow (denoted as Decision I) the lytic response (*left*) or the lysogenic response (*right*). The resulting lysogenic cell carries a repressed prophage shown in orange. The prophage is irreversibly induced only when a threshold amount of DNA damage causes an SOS response, leading to lytic development (Decision II). The prophage is normally extremely stable and rarely undergoes this type of induction by random DNA damage to produce progeny phage in a very small fraction of the lysogenic cell (basal or spontaneous induction). Some of the spontaneously induced cells enter the lytic cycle abortively, lose the prophage (curing), and become nonlysogens (Decision III) (65).

killing their hosts. λ phage infecting an *E. coli* cell makes a decision to follow either a lytic or a lysogenic pathway (**Figure 1**). If the lytic pathway is followed, the phage replicates its DNA autonomously, expresses the morphogenetic genes, assembles virions, and lyses the host. If the lysogenic course ensues, a stable lysogen is established in which the prophage is integrated into the host chromosome with lytic gene expression turned off. The prophage DNA replicates as part of the bacterial genome during subsequent cell divisions, and confers immunity to the cell against infection by another λ. Treatment with DNA-damaging agents, which leads to an SOS response, causes the lysogenic state to irreversibly switch into lytic development, mimicking the lytic infection. Otherwise, the prophage state is extremely stable, rarely undergoing induction by DNA damage. Some of these rarely induced cells enter an abortive lytic cycle by losing the prophage and becoming nonlysogens (curing). Similarly, after infection abortive lytic or lysogenic events may also occur (62). The importance of these abortive events has not been thoroughly studied.

GENE ORGANIZATION AND REGULATION

The λ genetic map and transcription profile involved in early developmental processes are shown in **Figure 2**. The gene organization of lambdoid phages is based on a number of recurring principles. Phage λ and its many relatives have genomes that evolved as highly mosaic, modular structures. This property has long been recognized and led to the formulation of the "modular genome hypothesis" (10, 14, 38, 101). A short summary of the λ phage modules and submodules is given in **Table 1**. Thus, for example, it is possible to replace the "immunity" module of λ by that of another lambdoid phage. The organization of the gene modules allows a typical cascade of

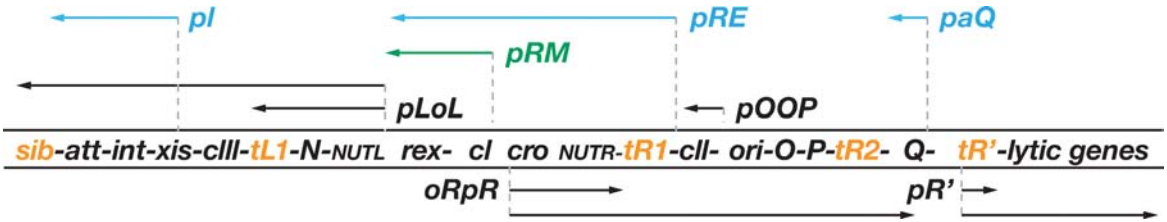

Figure 2

Genetic map and transcriptional units of the phage regulatory region. Key genes and signals discussed in the text are shown in their map order between the parallel lines. The early transcripts, the extended delayed early transcripts, and the late transcripts are shown in black arrows. The transcripts initiated from the *pI*, *pRE*, and *paQ* that are required for lysogeny are shown in blue. The *pRM* promoter, which is activated by the CI regulator, is required for maintenance of the lysogenic state, which is shown in green. Critical transcription terminators are marked in orange, including the *sib* region containing the *tI* terminator. Leftward promoters are indicated above and the rightward ones below the map. *pL* and *pR* are the early promoters and *pR′* is the late lytic promoter. The role of the *pOOP* promoter is not fully understood. The operators *oL* and *oR*, cognate to *pL* and *pR* respectively, are also shown. The immunity module of the λ chromosome encompasses *pLoL*, *rex*, *cI*, *oRpR*, and *cro*. *ori* is the origin of O- and P-mediated phage DNA replication (**Table 1**). Int carries the site-specific recombination reaction, and Int and Xis support the excision reaction.

phage gene expression in lytic growth delineating the early, delayed early, and late stages of transcription. This modular and temporal expression facilitates the alternative λ developmental pathways. Because of the transcriptional cascade, the repression of the early phage promoters *pR* and *pL* prevents expression of all lytic genes (**Figure 2**, **Figure 3**). In a prophage, this repression is carried out by the phage CI protein, which acts by binding to the *oL* and *oR* operators that overlap the *pL* and *pR* promoters, allowing the maintenance of the lysogenic state to be governed by CI alone; when CI is inactivated, e.g., by the SOS response, the lytic development follows. By blocking the *pL* and *pR* promoters of an incoming phage genome, the CI regulator confers immunity against further productive infection by another λ (superinfection immunity).

Table 1 A list of phage λ modules

Module	Genes and sites	Function
CI regulator	*pRM-cI*	CI activates *pRM*
Immunity	*pLoL-rex-cI-pRM-oRpR-cro*	CI represses *pL* and *pR*
CII regulator	*cII-pRE*	CII activates *pRE*
Site-specific recombination	*xis-int-att-tI*	Int and Xis catalyze recombination
General recombination	*gam-exo-bet*	Control and catalyze recombination
DNA replication	*ori-O-P*	Control of initiation of DNA replication control
Early antitermination	NUTL-*N-tL1*	N-antitermination and translational control
Late antitermination	*Q-pR′-tR′*	Q-antitermination for late gene expression
DNA packaging	*cos-Nu1-A*	Cleavage of head-full genomes
Head genes	*Nu1* to *FII*	Morphogenesis of head particles
Tail genes	*Z* to *J*	Morphogenesis of tails

Description of the functions of genes not discussed in this review can be found in (38).

The Lytic Transcription Cascade

The gene expression cascade that leads to the lytic mode of growth is the default mode of λ development. It is carried out in three stages. (*i*) Transcription is initiated with the synthesis of the early transcripts from the *pL* and *pR* promoters (**Figure 2**). Early transcripts, which encode two regulators, N and Cro, are attenuated at the *tL1* and *tR1* terminators, respectively. These transcriptional terminators play an important role in controlling the cascade of gene expression. By acting as a weak repressor for both *pL* and *pR* promoters, Cro facilitates the lytic mode (described below). The N protein is an antitermination factor that promotes the assembly of a transcription complex (9, 35). This assembly occurs on the RNA at the *nutL* and *nutR* sites and is made up of RNA polymerase and a number of host proteins called Nus. The N- and Nus-modified RNA polymerase can overcome the *tL1* and *tR1* transcription terminators, resulting in expression of the distal delayed early functions (30, 84). (*ii*) The delayed early functions include the lysogenic regulators CII and CIII, as well as the lytic DNA replication functions O and P, and the late gene regulator Q. (*iii*) After sufficient accumulation, the Q protein modifies RNA polymerase that has just initiated transcription from the *pR′* late promoter (66). This modification causes the RNA polymerase to become resistant to transcription terminators present downstream to *pR′*, allowing the expression of the late genes, which encode proteins for phage morphogenesis and host cell lysis. There is a kinetic separation between the expression of delayed early and late genes. This is caused by the location of the *Q* gene at the very end of the delayed early cascade and the high threshold level of Q protein needed for its activity (52, 63, 109). During the late stage of the cascade, the late gene products assemble phage virions and lyse the host. A similar temporal lytic cascade of gene expression follows prophage induction (38).

The Lysogenic Process

The lysogenic pathway is also initiated during the transcription cascade of the delayed early genes. Under conditions leading to lysogeny, the expression of the lytic regulators fails because Q is actively switched off by the accumulation of the critical lysogenic regulator CII, thereby blocking the default lytic pathway and switching to the lysogenic course. As discussed in greater detail below, an accumulation of the CII protein above a threshold level is critical for initiating the lysogenic switch. During this active switching from the default lytic to the lysogenic mode, CII stimulates the synthesis of Int, which catalyzes the insertion of the phage DNA into the host chromosome, and of the CI regulator, which binds to *oL* and *oR* to repress the early promoters. CII also inhibits Q function (see 52). These three activities are mediated by transcription activation of three promoters, *pI*, *pRE*, and *paQ*, respectively (**Figure 2**; see **Figure 6** below). Activation of all three promoters is critical for the establishment of a stable prophage state. Once the prophage genome integrates into the bacterial chromosome and CI protein represses *pL* and *pR*, the lysogenic state is established. The prophage state is extremely stable and is maintained through many generations of division (8). How CI repressor synthesis is maintained in the absence of CII is discussed later.

THE PROPHAGE STATE AND ITS MAINTENANCE

In the prophage state, the CI regulator controls the expression of three promoters. It represses transcription from the *pL* and *pR* promoters and positively and negatively regulates its own synthesis from the *pRM* promoter (**Figure 3**; see **Figure 6** below). In the prophage state, *pRM* is responsible for CI synthesis; CI expression from *pRE* is prevented owing to repression of CII in a lysogen.

Figure 3 illustrates a set of cooperative interactions of CI binding to DNA, which lead

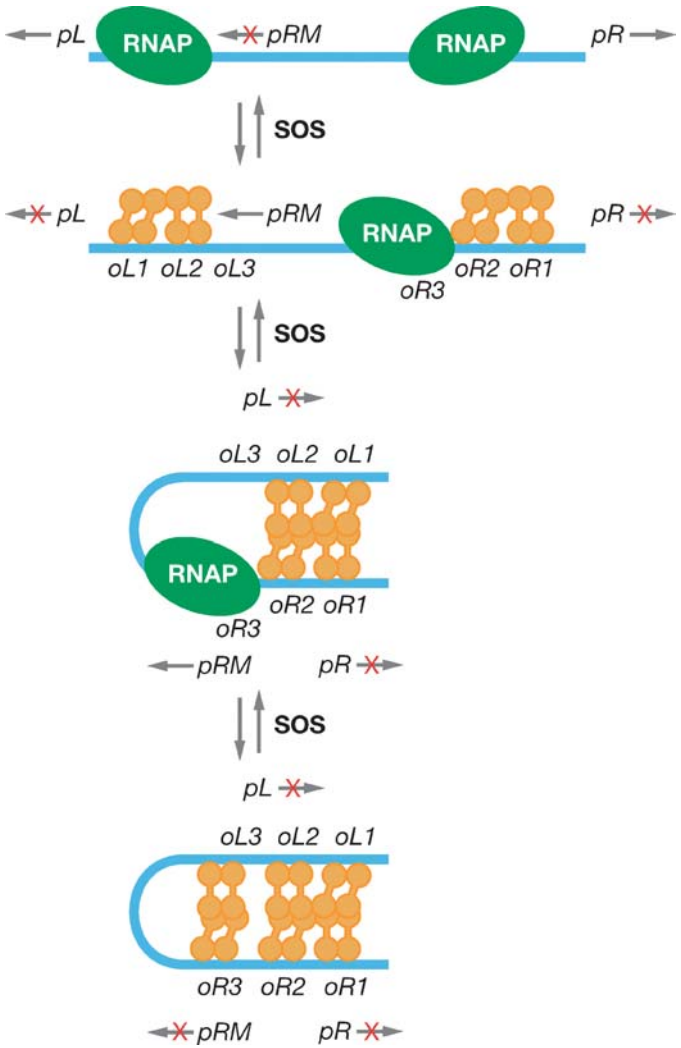

Figure 3

A scheme showing the repression of *pL* and *pR* by CI induced DNA looping [adapted from (24, 82)]. RNA polymerase is symbolized as a green ellipse; CI regulator monomers (depicted as orange dumbbells) have two domains: the N-terminal DNA binding domain and the C-terminal oligomerization domain. The top line shows a linear arrangement of the DNA. In the absence of CI, transcription is initiated at both *pL* and *pR* (*top line*). The second line shows how repressor molecules bind cooperatively to *oL1/oL2* and to *oR1/oR2* prior to DNA looping repressing *pL* and *pR* and activating the *pRM* promoter. The third line shows the first stage of DNA looping. RNA polymerase bound to *pRM* either before (*second line*) or after (*third line*) looping occurs transcribes *pRM*. Finally, further stabilization of DNA looping occurs by binding of CI to *oL3* and *oR3* together with repression of *pRM* when CI levels become very high (*fourth line*). The additional CI molecules bound to *oL3* and *oR3* interact to form a tetramer. The SOS response reverses the repression process (see text).

to extremely efficient repression (25, 64, 82). The CI repressor is made of two domains tethered by a short linker, an N-terminal DNA binding domain with a helix-turn-helix motif, and a C-terminal oligomerization domain. The intrinsic relative affinities of CI to its operators at *oR* and *oL* are as follows: *oR1* > *oR2* > *oR3* and *oL1* > *oL2* > *oL3*. Dimers of CI cooperatively bind to *oL1* and *oL2* on the left and *oR1* and *oR2* on the right by forming tetramers repressing *pL* and *pR*, respectively. Furthermore, another cooperative interaction between these two sets of tetramers bound to *oL* and *oR*, 2.3 Kb apart, leads to the formation of a DNA loop held by a CI octamer, i.e., two interacting tetramers, that enhances repression. In this DNA-multiprotein complex, the CI dimer bound at *oR2* also stimulates *pRM* transcription, thus activating CI synthesis in a repressed prophage by a positive autoregulatory loop (44, 75). As the CI concentration increases because of *pRM* activation, two additional CI dimers are recruited to bind at *oL3* and *oR3* to further stabilize the *oL-oR* loop. In this context, the binding of CI to *oR3* represses *pRM* and prevents CI overexpression. Based on direct measurements of CI (86), recent calculation shows that a lysogenic cell contains about 250 CI monomers when the cells are growing in rich media (23). This translates into about 30 dimers per prophage copy, assuming an average of four chromosomes per cell. This number of CI molecules not only achieves a strong repression of the phage promoters but also sets the threshold level for SOS induction. Note that cell division does not randomly reduce the CI concentration to a point low enough to cause prophage induction (8).

Repression of transcription from the *pR* promoter inhibits not only expression of genes in that operon but also phage DNA replication by preventing "transcriptional activation" of λ *ori*, the site where phage DNA replication is initiated (26, 33). This inhibition occurs even if O and P functions are present. (71, 106). This regulation appears to be critical for establishing a lysogen. If the λ origin

replicates after the phage is integrated because O and P are still available, the replication event is lethal to the cell, resulting in abortive lytic infection (11). Thus, it is important to immediately block phage replication as the choice for lysogeny is made.

THE DEFAULT LYTIC COURSE: Cro AND N FUNCTIONS

The gene coding for Cro, which is the essential lytic regulator, is the first one to be transcribed from the *pR* promoter following phage infection or prophage induction. It is a weak repressor of the *pR* promoter, allowing continuous expression from *pR* during lytic infection (29, 94, 95). Cro is a single-domain protein that binds as a dimer to the *oL* and *oR* operators (60, 82). The intrinsic affinity of Cro for the individual operators is $oR3 > oR1 \geq oR2$, and, unlike CI, higher-ordered structures, i.e., tetramers or octamers of Cro have not been detected (19). However, Ackers and coworkers found a small amount of cooperative binding of Cro dimers to adjacent sites in the *oR* complex (19). Whether such cooperative binding is due to dimer-dimer interaction or to changes in DNA needs to be investigated. It was shown, however, that changes in DNA accompany the cooperative binding of CI (21).

The higher affinity of Cro binding to *oR3* supports the hypothesis that during lytic development Cro binds first to *oR3*, repressing transcription of the *cI* gene from the *pRM* promoter (45). It was therefore proposed that Cro binding to *oR3* represses *pRM* and completes the switch to the lytic pathway as well as sustaining it after an initial SOS-mediated inactivation of CI in a lysogen (28, 46, 74). However, recent experiments suggest that the presence of Cro might be unimportant for the lysogenic to lytic switch during induction of the prophage (94). Furthermore, Little and coworkers showed that replacing *oR3* by the weaker *oR1* Cro-binding site has a marginal effect on prophage induction (64). Thus, it appears that the critical role of Cro in lytic development may be only to turn down the *pL* and *pR* promoters and that its role in turning down *pRM* is not critical but supplementary (52, 94). An argument that Cro binding to *oR3* sets the lytic course has also been made to explain the role of Cro following phage infection (82). This interpretation of the role of Cro is also unlikely because a phage carrying the same *oR3* to *oR1* variant (see above) that reduces Cro but allows CI binding still shows lytic growth (64). However, under certain conditions Cro binding to *oR3* may contribute to lytic growth after prophage induction. The function of Cro in lytic development is addressed below.

The N Antiterminator

The N protein, like Cro, is a critical lytic regulator but acts by a completely different mechanism. It antiterminates transcription at termination signals, allowing expression of distal genes in the *pL* and *pR* operons. N protein, once made, binds to the specific RNA sites NUTL and NUTR. The NUTL site is located between the *N* gene and the *pL* promoter, and NUTR is downstream of *cro*, the first gene in the *pR* operon (**Figure 1**). The Nus factors, a complex set of host proteins, take part in cellular transcriptional and translational processes and interact with the N system (18, 93). Purified in vitro studies have defined NusA, NusB, NusE, and NusG as the host components of N-antitermination (22, 68). Each NUT site can be divided into two parts, BOXA and BOXB (32). BOXB is a stem-loop structure in the RNA and is specific for N binding (16). As it binds BOXB, N associates with NusA, NusG, and RNAP. BOXA RNA is specific for NusB and NusE binding (77, 80). It is proposed that N, Nus factors, and Nut interact and complex with RNA polymerase while tethered on the same RNA (76, 102, 104, 105). Although N is the essential factor for antitermination, NUT and the Nus factors confer stability and full activity to the antitermination complex (22, 85).

N is a critical regulatory protein of phage λ, which is reflected in the number of

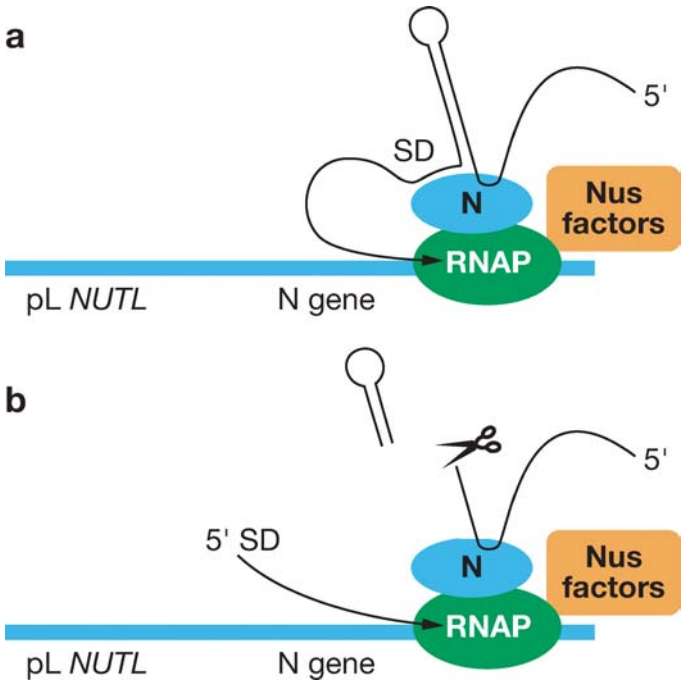

Figure 4

Models for N-mediated translation repression. The complex of RNAP is modified by *NUT*, N, and Nus factors to form an antitermination complex. Translation repression of N takes place on the uncleaved RNA in the absence of RNase III action (*a*). In the presence of RNase III cleavage occurs and the Shine–Dalgarno (SD) sequence for N is no longer held in close contact to the antitermination complex, translational repression is prevented, and efficient expression of N can take place (*b*).

ways its expression is controlled. At the transcription level, *N* is regulated from *oLpL* by CI- and Cro-mediated repression. At the translation level, N represses and autoregulates its own translation, and the endoribonuclease RNase III modulates this repression (**Figure 4**). By acting at their respective sites located upstream of the N gene, N and RNase III control N translation (103, 104). High concentrations of RNase III as found in cells grown in rich media prevent N-mediated translation repression altogether, whereas low concentrations of RNase III found in cells grown in poor media allow translation repression. At the posttranslational level, the Lon protease degrades N causing a relatively short half-life.

N expression from the *pL*-antiterminated transcripts is dependent upon the level of RNase III in cells. If RNaseIII concentration is high, processing of the RNase III site (RIII site) between NUTL and the N message is rapid, causing high rates of N translation. If RNase III concentration is low or zero, processing of the RIII site occurs slowly if at all, allowing repression of N translation. Cellular RNase III concentrations rise and fall with growth rate (12), and correspondingly, N translation rises and falls with growth rate (104).

Evidence suggests that the N translation repression complex is dependent on *E. coli* RNAP and the N-transcription antitermination complex (105). Under conditions of limiting RNase III, the antiterminating complex prevents translation of N from its antiterminated message. This means that N is expressed from RNA transcripts that terminate at *tL1*, i.e., do not form an antitermination complex. Thus, under minimal growth conditions where RNase III activity is limiting, N concentrations would be stringently controlled by negative autoregulation. On the other hand, in rich media, RNase III processing would ensure high N concentrations. Viral lytic development predominates in rich media, and lysogenic development is enhanced under limiting growth or starvation. High N concentration enhances lytic growth of the phage, as evidenced by λ forming clear plaques on cells expressing N from a plasmid (104). Clear plaques indicate that only few cells follow the lysogenic pathway. Low levels of N are made following λ infection of cells grown in poor carbon conditions; these same conditions have been shown to enhance lysogen formation following infection at low multiplicity of infection (56). These latter conditions are likely to reduce Q concentrations and provide a better opportunity for lysogeny. Regulation of N also appears to be a way in which λ senses environmental conditions through RNase III and responds by increasing or decreasing N concentrations and altering the lytic/lysogenic response. We note, however, that at high

multiplicity of infection, there is no effect of growth media on lysogenization efficiency (27).

SWITCHING THE DEFAULT LYTIC MODE TO LYSOGENY: ROLE OF CII

The inhibition, or absence, of lytic functions is not sufficient for the switching to the lysogenic mode. Rather, as noted above, infection resulting in a lysogenic response proceeds through a number of required events: integration of the DNA, efficient repression of the early promoters, as well as timely inhibition of the lytic genes expression. These requirements are met by CII turning on *pI*, *pRE*, and *paQ* promoters to express Int and CI, and to inhibit Q function, respectively. All three promoters contain a direct repeat TTGC-N6-TTGC sequence that binds to CII. The N6 region corresponds to the –35 elements of these promoters. Expression from all three CII-activated promoters is coordinated by the CII protein during infection. However, the mechanism of activation of the promoters by CII is not well understood. Specific mutations in the α or σ subunits of RNAP prevent CII-mediated activation from these promoters in vitro (48, 67). Consistently, these RNA polymerase mutants prevent the establishment of lysogeny in vivo (78).

The quaternary structure of the CII protein has been solved recently (20a, 81a). The structure shows that a CII tetramer is made of two nearly equivalent dimers. Each of the four monomers contains a helix-turn-helix DNA-binding motif but only two of the monomers appear, by modeling, to be involved in actual DNA binding at the direct repeat sequence. The function of the other two helix-turn-helix motifs in the tetramer is not known. The direct repeat sequence of *pRE* is located within the N-terminal coding sequence of CII. Incidentally, in an elegant genetic study, Friedman and coworkers isolated and analyzed a CII mutant defective in transcription activation of the *pE* promoter in lambdoid phage P22 (88). This mutation affects the N-terminal sequence and is not in the helix-turn-helix domain of CII. This mutation also modified the *pRE* promoter so that CII can activate the mutant *pRE* but not the wild-type *pRE*. This suggests that this N-terminal sequence of the protein may play a role in determining the specificity of CII binding to DNA (88).

The *pI*, *pRE*, and *paQ* promoters are located within the Xis, CII, and Q protein coding sequences, respectively. The Xis protein is needed for excision of the prophage DNA from the chromosome after induction. For rapid synthesis of Int, which facilitates integration after infection, CII activates the *pI* promoter. The integration reaction also requires the integration host factor, IHF. IHF is critical for generating the multicomponent Intasome structure, which catalyzes the integration reaction (38). Since *pI* is located within the *xis* gene, the *pI* transcript synthesizes Int but not Xis, helping to ensure that integration will not be accompanied by the presence of Xis function. By the same criterion, CII activates the *pRE* promoter to direct rapid synthesis of the CI regulator following infection. The amount of CI made from *pRE* during lysogenic response was found to be as much as 10- to 20-fold higher than the amount made from *pRM* in an established lysogen (86). The initial high concentrations of CI may guarantee that all infecting and replicating phage genomes become repressed. But the CII-dependent *paQ* promoter, which lies within the Q gene, was found to reduce Q function (52), providing a mechanism by which CII reduces late gene expression to enhance lysogeny (17, 70). Mutations affecting the ability of the *pRE* or *paQ* promoter to respond to CII prevent the lysogenic response resulting in clear plaque formation (42, 52).

Regulation of CII Activity

CII, which plays a key role in the lysis-lysogeny decision, is regulated at numerous levels (**Figure 5**) (39, 43, 50, 83, 91): (*i*) The transcription of the *cII* gene is inhibited both

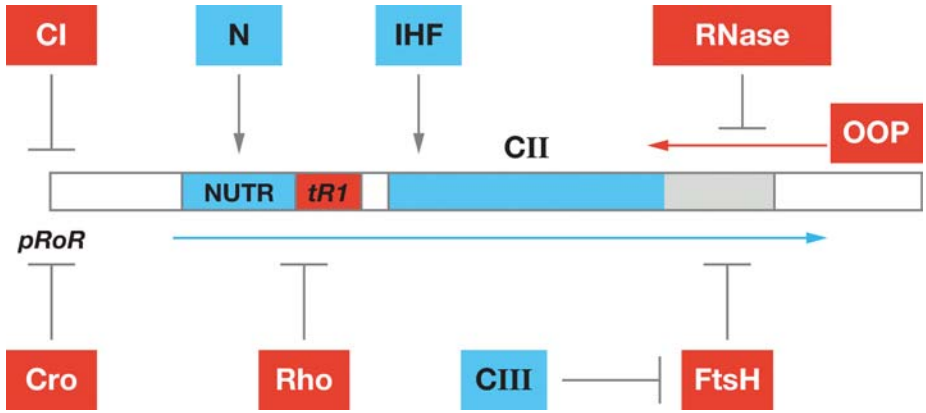

Figure 5

Multilevel regulation of CII activity. CI and Cro negatively regulate the CII gene at transcription initiation. *tR1* aided by Rho factor reduce transcription elongation into the CII gene. The N antitermination factor allows the extension of transcription beyond *tR1*. IHF stimulates CII translation initiation. The antisense OOP RNA together with RNase III reduces CII mRNA stability, and FtsH protease acting at the C terminus of CII is responsible for the rapid proteolysis of CII. The center bar represents the DNA; the CII gene and the positive controls are shown in blue and negative controls in red. The direction of CII and OOP transcriptions are shown in blue and red, respectively.

by Cro and CI binding to *oR*, and is stimulated by the N antitermination factor acting at *NUTR*. High rates of CII synthesis take place only for a limited period before being repressed, by Cro or CI. (*ii*) Translation initiation of CII is stimulated by IHF (43). An IHF binding site is located immediately upstream of CII, which has been proposed to stimulate CII translation in the presence of IHF by an unknown mechanism (72a, 80a). (*iii*) The stability of CII mRNA is affected by the OOP RNA, a short antisense transcript complementary to the 3′ end of the *cII* mRNA (57, 58). RNase III recognizes and cleaves the CII-OOP double-stranded RNA, thereby initiating rapid CII mRNA degradation (57). The DNA coding for the protease target is also the target of CII mRNA degradation mediated by the antisense OOP RNA. It was reported that the stability of the OOP RNA is reduced by polyadenylation but whether this process regulates CII concentration has not been clarified (108). (*iv*) The ATP-dependent protease FtsH is responsible for the rapid degradation of CII (39, 50, 91). Host mutations in *ftsH* lead to stabilization of CII and thereby an increased lysogenization frequency. A C-terminal flexible tail of CII, which is not required for CII activity, acts as a target for initiating rapid CII proteolysis by FtsH (20, 51). (*v*) The long leader CI RNA initiated from *pRE* is antisense to *cro*, which could prevent the translation of Cro (92). Indeed, an *ftsH* host mutant that results in higher concentrations of CII was found to be defective in Cro (79, 83, 96). Unfortunately, the concentration of Cro as a function of *pRE* activity has not been directly measured. (*vi*) CIII, which is a 54-residue long peptide and required for lysogeny, controls the rate of CII degradation by acting as an inhibitor of the FtsH protease (40, 51, 53).

Regulation of CIII

The *cIII* gene expression is also subject to multiple controls by λ CI, Cro, and N and by the host RNase III (**Figure 5**). CI and Cro inhibit CIII synthesis by binding to *oL*, and the N-antiterminator stimulates CIII expression by acting at *NUTL*. Unlike its negative effect on CII, RNase III stimulates CIII translation.

It was shown that the mRNA that codes for the amino terminal residues of CIII is present in two conformations (3, 54). In one conformation, the translation initiation region is occluded, preventing *cIII* translation. In the other, the mRNA is open allowing efficient translation. Point mutations that favor one or the other structures have been described. It appears that RNase III regulates *cIII* translation by acting as an RNA chaperone to affect CIII mRNA structure without processing (2).

THE DECISION PROCESS: GENETIC FUNCTIONS AND EXTERNAL INFLUENCES

We now summarize the critical events that take place in the decision making process following infection by λ (Decision I in **Figure 1**, and shown schematically in **Figure 6**). Following infection, λ begins a cascade of transcription destined for the lytic mode. Under appropriate conditions the lytic cascade can be switched off, allowing entry into the lysogenic pathway. The switch requires high threshold concentrations of CII, which facilitates CI and Int synthesis and reduces the regulator function Q. CI, once made, maintains the repressed state of the prophage. In establishing repression, CII and CI act temporally to inhibit lytic functions. CII acts first by direct reduction of Q function, and as CII function ceases, CI takes over by repressing the *pR* promoter. CI executes repression after the CII-mediated switch and appears not to participate in initiating the switch. The activity of CII is programmed to allow an initial overshoot of CI expression from the *pRE* promoter and a preemptive inhibition of Q activity (52, 86). The overshoot may be required to ensure the repression of all lytic genes of the infecting and replicating phage genomes in the cell during the establishment of repression. Repression of *pR* by CI at this stage also ensures immediate cessation of phage DNA replication by preventing transcription through the *ori* site (97).

Following infection, transcription from early *pL* and *pR* promoters would start the lytic pathway by default with N antitermination of transcription and subsequent expression of Q. Q in turn antiterminates transcription, leading to late lytic gene expression, cell lysis, and phage release. To set the course for lysogeny, CII reduces Q function in two ways. First, CII activates antisense *paQ* RNA inhibiting Q. Second, CII activates *pRE* for synthesis of CI, which represses *pR* and thus Q transcription. CII continues to repress Q expression via *paQ* until *cII* transcription is repressed by CI at *pR*. This kinetic coordination of Q shutoff by CII and CI ensures the switch from the default lytic pathway to lysogeny. Q protein must build up to a high threshold concentration to become functional, thus providing a window for CII to exert its effect on Q through *paQ*. In this way, CII prevents Q from reaching its threshold concentration. If CI action is prevented, CII inhibition of Q via *paQ* is not sustained because of repression of CII by Cro acting at *pR* and by rapid CII degradation. Concordantly, λ phage mutants defective in CI, *oR*, or CII follow exclusively lytic growth, whereas mutants defective in Cro are unable to follow the lytic pathway.

Multiplicity Effect

The lysis-lysogeny decision is influenced by the number of phage particles infecting the cell as well as by the cell physiology (55, 56). High multiplicity of infection favors lysogeny. Direct measurements of CII activity showed, in wild-type infection, functional CII activity in cells infected with two or more phages (52). This threshold level of CII is reached when two or more phage infect a cell. In single infection this critical level of CII is not attained, as mentioned above, allowing lytic development.

Physiological Effects

In cells grown in rich media the lytic course predominates, whereas growing cells in poor

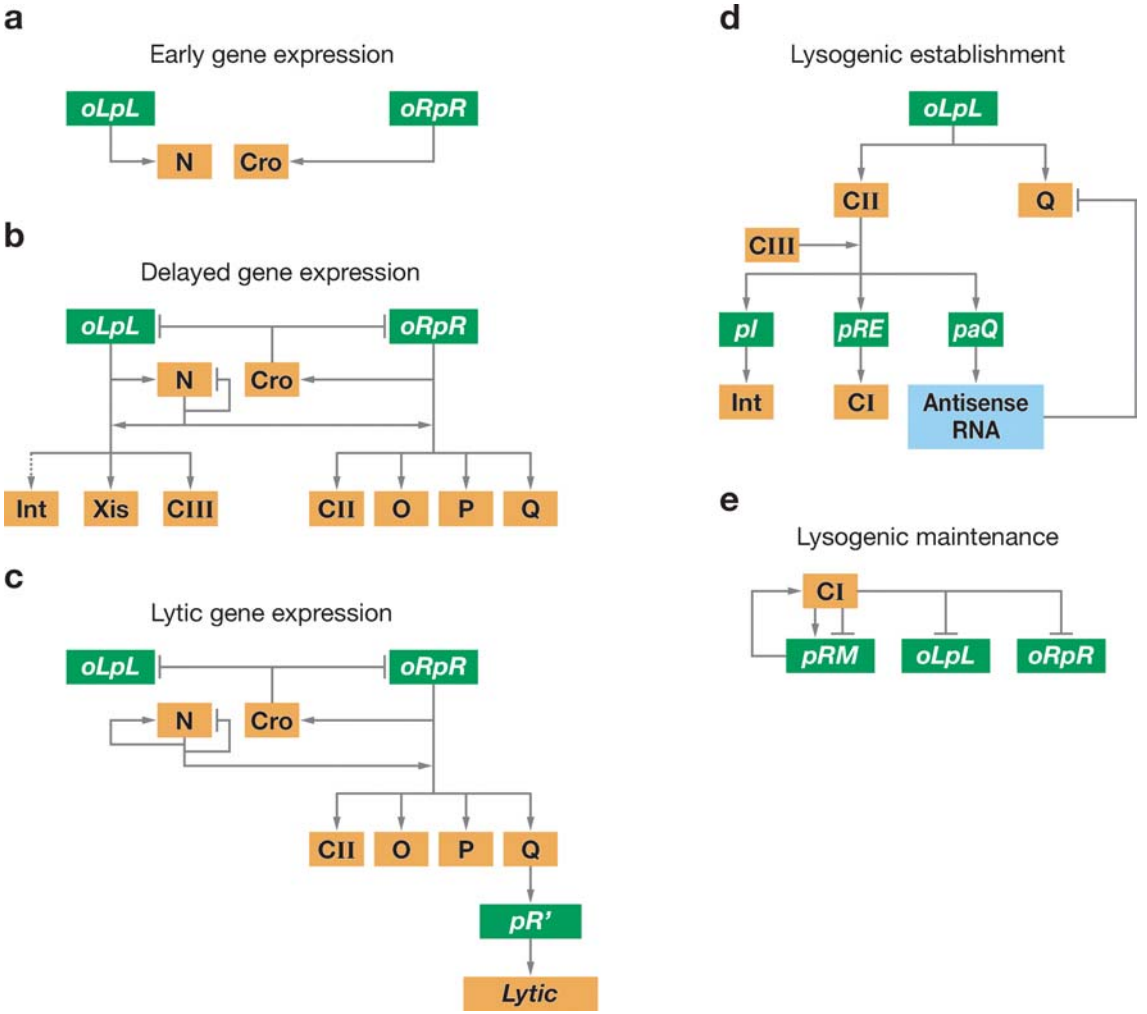

Figure 6

Description of the λ lytic and lysogenic genetic network. Arrows mark the positive effects between elements that make up the genetic network, whereas bars denote inhibitory effects. Promoters and operators are shown in green, the phage genes in light orange, and *cis* antisense RNA is shown in light blue (Cis acting has been used to note that it acts on the RNA from the opposite strand and not on an RNA originating from different sequences in the genome). Arrows emanating from the promoters denote transcription of specific functions. The OOP antisense RNA and the yet unknown threshold effect of Q activity are not shown. For simplicity, the developmental network is divided into early gene expression (*a*), delayed gene expression (*b*), lytic gene expression (*c*), lysogenic establishment (*d*), and lysogenic maintenance (*e*). The dotted line in (*b*) leading to Int expression signifies reduced level of Int expression due to retroregulation of *int* mRNA.

media increases the chances of the lysogenic pathway after single infection (56). Cells growing in rich media have higher concentration of the host global regulator RNase III, which leads to high rates of expression of the protein N favoring lytic growth [see (104) and references therein]. In carbon-starved cells, on the other hand, RNase III and consequently N concentrations are low. Under these conditions N translation is repressed.

This reduction of N concentration would reduce Q expression to a level that provides more opportunity for lysogenic response. Nevertheless, when cells are infected by two or more phages, CII function is epistatic to the effects of growth conditions preferring lysogeny.

Unlike the decision that λ makes after the infection process described above whereby infected cells follow either lytic or lysogenic development, prophage induction leads exclusively to the lytic course (Decision II in **Figure 1**). It was proposed that Cro is responsible for repressing CI expression following induction to keep the developmental switch in the default lytic mode (82), although the action of Cro in keeping the lytic course on track is simply to reduce the concentration of the unstable master lysogenic regulator CII.

COEXPRESSION OF BOTH LYTIC AND LYSOGENIC GENES FROM THE SAME OPERON: A PARADOX

The *pL* operon encodes a large number of ORFs of which only N, CIII, Xis, and Int are essential for either the lytic or lysogenic response (see **Figure 2**). N is a key regulator for lytic growth of the phage. On the other hand, the lysogenic function CIII acts as an inhibitor of a host protease (FtsH, also called HflB) that destabilizes the critical lysogenic regulator CII (40, 47, 51). Proteins Int and Xis carry out site-specific recombination (59, 73). During lysogenization, Int catalyzes the integration of the phage genome into the bacterial chromosome site, *att*, whereas Int together with Xis catalyze the reverse reaction to excise the λ chromosome during prophage induction. The *pR* promoter also transcribes both lytic and lysogenic genes, *cro*, *cII*, *O*, *P*, and Q. Whereas Cro is another critical regulator for lytic growth of the phage, the very next gene in the operon encodes the CII protein, which coordinates the lysogenic pathway. O and P are needed for phage DNA replication prior to morphogenesis, whereas the second lytic regulator Q activates the synthesis of proteins for phage morphogenesis and cell lysis. Thus functions necessary for lysogenic and lytic development are both expressed from the *pL* and *pR* promoters. The location of both lysogenic and lytic genes in the same operons creates an apparent paradox in our mind about the lysis-lysogeny decision. This paradox is resolved by regulating the synthesis and degradation of critical RNA and proteins. This regulation provides the catalytic or stoichiometric amounts of functions as required to pursue either the lytic or lysogenic course. As examples, during lytic response, the CII regulator is rapidly eliminated by proteolysis, whereas in the lysogenic response, CII accumulates and limits Q activity. Presumably, the high threshold requirement for Q function allows the infected complex a time window for controlling the concentration of CII.

In the lysogenic response, high concentrations of Int are made from pI under the control of CII, while the expression of Int from the *pL* is greatly reduced by retro-regulation because of Int mRNA degradation by RNase III from a site called *sib*, located on the other side of *att* (see **Figure 2**) (36). Prophage induction leads to the expression of both Int and Xis from the *pL* promoter because the *sib* regulator is detached from the *int* gene in the prophage DNA.

THEORETICAL STUDIES

The lambda genetic network has been a fertile ground for theoretical modeling of decision-making processes during the regulation of development, and for testing new modeling methodologies of genetic networks in general. Models have been constructed addressing the lysis-lysogeny decision in terms of the lambda genetic switch describing the competition between CI and Cro, using statistical mechanics with the explicit goal of obtaining bistability. The earlier models focused on the (*i*) probabilities of the occupancy of the *pR* and *pRM* promoters by different binding configurations of CI and Cro, and of *pR* and *pRM* by

RNAP, and (*ii*) Gibbs free energies of binding characterizing the different configurations as free parameters (1, 87, 90). These models assumed that the behavior of the reactants studied under in vitro conditions reflects in vivo situations, and did not incorporate the possible existence of other levels of regulation, e.g., regulation of translation of CI and Cro, degradation of CI and Cro, and anticooperative binding of the two proteins to adjacent operators (87).

More recent models, based on the same approach, also incorporated stochastic effects in the concentrations of the regulatory proteins and, as a result, in the selection between lysis and lysogeny (5, 7, 49, 69, 89, 98, 100, 110). Although apparent agreement between the theoretical calculations and the experimentally observed values was noted, these efforts did not lead to a theoretical description with improved predictive values. However, the theoretical analysis of the exceptional stability the prophage state of λ led to the conclusion, now confirmed by experimental evidence, that a view of the switch focusing only on oR to explain the stability is incomplete, and that oL participates as well (7, 23).

An advantage of computer simulations is their ability to take into account multiple elements and variables within the decision module of the λ network (5, 49, 69). Furthermore, they can predict, for example, the values of reactant concentrations during the temporal execution of the lytic and lysogenic pathways, which can be readily compared with biological experiments. Nevertheless, at present there are only limited experimental data on the kinetic changes in the concentration and activity of the regulatory elements in the network and the strength of their interactions, which are crucial to achieve real agreement between theoretical results and experimental observations. Furthermore, the in vivo values of the parameters may differ quantitatively by orders of magnitude from in vitro ones owing to such factors as macromolecular crowding, variation in local concentrations, and yet unknown functions, as alluded to above. By exposing inconsistencies with the observed behavior, future theoretical studies with predictive values are expected to play a more important role.

OPEN QUESTIONS, SUMMARY, AND CONCLUSION

Counting Infecting Phage

The decision made by a phage-infected cell is dependent on the multiplicity of infection. When one phage infects, most infection shows lytic development. However, when two phage infect, the lysogenic pathway prevails. What is the molecular basis for such a dramatic response to a small change, which to some may be counterintuitive? Furthermore, the network response also suggests tight communication between two coinfecting phage genomes. This suggests that replicating phage genomes after single phage infection have little effect on the decision process. This issue requires further investigation. However, there are conditions when a single infecting phage enters the lysogenic pathway. When infecting an *hflA* or *hflB* mutant host, efficient lysogenization takes place. As discussed, these mutant hosts increase the level of CII function.

A possible parsimonious model to explain the multiplicity response runs as follows: First, multiple infection results in the titration of a critical regulatory host factor that is present at a very low concentration. One such candidate is FtsH, the product of the *hflB* gene of which there are less than 100 molecules per cell (99). Second, the phage CIII protein acting as an inhibitor of FtsH plays a critical role in the decision. Indeed, mutants that result in a elevated CIII translation (by about threefold) no longer respond to the multiplicity of infection and can efficiently lysogenize upon single infection (4; A. Rattray, unpublished). Thus, we expect that a small increase in multiplicity from one to two would raise CIII to concentrations that inhibit FtsH increasing CII expression to allow lysogenic development.

The Distinction Between Phage Infection and Prophage Induction

The idea that two infecting phage are critical for switching on the lysogenic course raises interesting questions. A lysogen contains a number of host chromosomes at various stages of replication, and accordingly more than one prophage. Would induction result in high CII activity as is found in multiple infections? If a high concentration of CII accumulates, we would expect inhibition of Q function by CII, even after CI inactivation by induction. It remains to be seen whether such high concentrations of CII if they occur would affect Q function and thus reduce lytic phage yield.

Role of Cro in Lytic Growth

An open question is the role of the high-affinity binding of Cro to the *oR3* operator site in lytic growth after phage infection or prophage induction. It is clear that Cro is essential to allow lytic development. Is this high-affinity binding critical to regulate *pRM* following prophage induction or does it have a critical role in lytic decision? This issue needs to be addressed by the use of phage mutants defective in oR3 that uniquely affect Cro binding and do not affect CI binding or pRM activity. Would such mutants affect lytic growth after phage infection or prophage induction? The intercalation of CI, Cro, and RNAP binding site at this locus may make the isolation of such phage mutants problematic.

Evolution of Temperate Phages

Phage λ lifestyles have been a textbook case for complex genetic control circuits. Gene order, modular construct, recruitment of specific host factors, and the presence of a complex genetic network allow the infecting phage to make decisions and to proceed through alternative developmental pathways. The lysis-lysogeny bistable decision is clearly much more complex than originally portrayed. This complexity was most probably reached through evolutionary forces tinkering with specific elements present in the individual functional modules that evolved to act in concert. Of interest is a recent approach of tinkering with λ modules that revealed the robustness of its genetic network (6, 72). Although the λ lysogenic promoter *pRM* can tolerate a number of mutational changes, we note that such tolerance may be limited to specific environmental conditions.

EPILOG

In summary, a small set of regulatory proteins in an organism as simple as a bacteriophage function through a diverse set of macromolecular interactions in a temporal fashion. Future investigations into the detailed molecular aspects of the functions of specific modules coupled with kinetic and quantitative analysis of the phage genetic network will yield a realistic picture of this important paradigm for more complex developmental processes (61).

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