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REPLICATOR ACTIVATION IN LAMBDA

WILLIAM F. DOVE, ERMILE HARGROVE, MASAKO OHASHI,
FINN HAUGLI, AND ARABINDA GUHA

McArdle Laboratory, University of Wisconsin, Madison, Wisconsin, U. S. A.

Autonomous replication of a genetic element is operationally characterized either by kinetic or physical independence, or by genetic dependence. Lambda illustrates all three of these criteria. (For reviews, see Echols and Joyner (1968) and Dove (1968).)

During replication of the virus genome after infection, molecules are found which consist of lambda DNA alone, often in a closed circular structure (Tomizawa and Ogawa (1968)). Viral DNA is made at an overall rate comparable to that for host DNA in the infected cells, so a particular viral sequence is replicated about 100 times more rapidly than any particular host sequence. Finally, the products of the viral genes N, O, and P are essential for this process.

In contrast, prophage lambda is integrated into the host chromosome. It is replicated at the same rate as any bacterial element, and requires only the action of the repressor gene *cI* to maintain lysogeny.

The replicon hypothesis of Jacob, Brenner, and Cuzin (1963) states that any genetic element capable of autonomous replication must possess an origin of replication (replicator) and gene(s) specifying product(s) (initiator(s)) which can specifically recognize and act on this origin to initiate DNA replication. The first condition seems to be an obvious one; it is less obvious that an autonomous element must carry at least one initiator gene of its own.

In this talk we shall summarize studies on the control of replication in lambda. In conjunction with work carried on in a number of other laboratories, our experiments have identified lambda-specific replication genes, a single replicator, and an unexpected phenomenon - that the lambda replicator is not active for initiation unless it is in a state of being actively transcribed.

THE EARLY CONTROL REGION OF LAMBDA

Let us first orient this discussion by reviewing a map of the region of the lambda DNA molecule in which lie all of the genes involved in replication and its control. This is shown in Figure 1.

Cistrons N, O, and P act in lytic growth. N produce a product which may serve a general stimulatory role in the transcription of pre-replicative and even post-replicative genes. O and P produce products which are essential for autonomous DNA replication, probably in a direct sense rather than by exerting control over other genes.

Cistrons cII and cIII control the decision between lysogeny and lytic growth after infection. They do this not by controlling viral DNA replication, but by preventing late events in lytic growth.

Cistron cI acts to stabilize the lysogenic state rather than to control the decision not to lyse. It does this by producing a repressor protein which, as Ptashne and Hopkins (1968) have so nicely shown, binds to two sites (operators) lying at either end of the cI

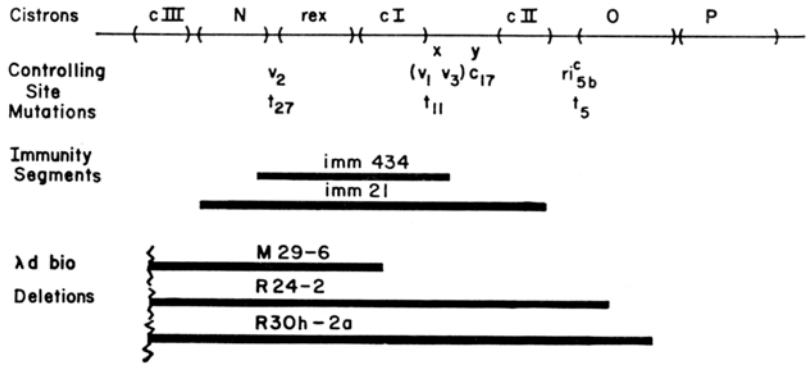

Fig. 1. The early control region of lambda.

A map of the region of the lambda linkage group in which lie all mutations known to control DNA replication and lysogeny. Cistrons, identified by recessive mutations, are indicated above the line. Regions x and y are defined by *cis*-acting mutations. Controlling site mutations are indicated and are discussed in text.

The regions replaced or deleted in immunity hybrids or in defective biotin transducing particles are indicated in heavy lines.

cistron and presumably controlling transcription to the left and to the right. The mutation v_2 acts as a constitutive allele of the left-hand operator; the double mutant v_1v_3 comprises a constitutive allele of the right hand operator.

The cistron rex acts in the lysogenic state to produce a product necessary for blocking the growth of rII mutants of T-even phages.

A number of absolute defective mutants, propagated in the prophage state, have been isolated by Fuerst and his colleagues. Mutants of this sort are indicated "t" and will be discussed.

Next, we have indicated the segments of lambda which are replaced in the hybrids λimm_{434} and λimm_{21}, wherein the immunity determinants of lambda are replaced by those of phages 434 and 21 respectively. Finally, we have indicated the extent of deletions associated with biotin-transducing phages studied by Kayajanian (1968). The immunity segments and biotin deletions have served to map unambiguously the mutations involved in the studies we shall report.

In schematic terms, the early control region of lambda has a structure

$$\text{—— N —— } (o_l p_l) \text{ —— cI —— } (p_r o_r) \text{ —— O —— P ——}$$

where o refers to an operator (a binding site for repressor) and p to a promotor (a binding site for RNA polymerase).

This early control region may control the action of the entire lambda chromosome by a process of sequential induction. Because the N product acts as a general inducing element, because DNA replication stimulates the action of a number of late-acting genes, and because the Q product is necessary for transcription of all late-acting genes, the early control region is able to direct all lytic activity in lambda.

THE REPLICATION GENES OF LAMBDA

Cistrons O and P are essential for autonomous replication under all known conditions. We have asked whether the products of these cistrons are specific for the lambda genome, or whether all lambdoid phages can complement lambda mutants defective in

O or P. We have found that only phage 434 can provide O function for lambda; phages 21, ϕ80, ϕ81, and 82 cannot. A homologous function for cistron P can be provided by phages 21, 82, and 434, but not by ϕ80. Thus cistron O is highly lambda-specific. Since the DNA of the lambdoid phages contains no unusual nucleotides in large number, one presumes that such a lambda-specific replication product acts in selecting the replication template (for example, at the initiation step) rather than in synthesizing precursors for DNA.

That the O and P products act at an initiation step rather than a termination step is suggested by the results of Ogawa and Tomizawa (unpublished) which show that density-labelled DNA from λsusO or λsusP undergoes no detectable density shift after infection on a non-suppressing host[1].

The ensemble of genes N, O, and P acts freely *in trans*. We have found that the triple *sus* mutant $\lambda\, \mathrm{susN_7O_{29}P_3}$ multiplies at least 20% as well as does a wild type helper after mixed infection of a non-suppressing host.

Two mutants, t_5 and t_{11}, isolated as absolute defective prophage mutants, show a non-complementable defect in autonomous replication. We have extended the observations of Green and Brachet (personal communication) on the multiplication of these mutants by means of a sensitive assay which we call a colony-sparing unit assay (Dove and McLeester, in preparation). This assay rests on the fact that any phage particle whose genome can express the wild type cI allele can spare a thermoinducible lambda lysogen from death during a brief exposure to elevated temperatures. The method and characteristics of this assay are described in the legend to Table 1.

In Table 1 are shown the burst sizes for various absolute defective mutants after induction and after infection, in the presence of wild type helper. The wild type prophage λ_{Y10} and the O-defective mutant t_{60} give large burst sizes both after induction and after infection. The mutant t_{27} shows a defect in phage yield after induction, but not after infection. Mutants t_{11} and t_5 do not give large bursts in the presence of helper

Table 1. Multiplication of defective mutants in the presence of helper

Phage	Burst size	
	After induction	After infection
$\lambda + Y_{10}$	18	25
λt_5	0.6	0.5
λt_{11}	0.5	1.0
λt_{27}	1.7	30
λt_{60}	10	20

Lysogens were infected with helper λcI_{857}, induced by ultraviolet irradiation, and allowed to grow. This lysate was then adsorbed to sensitive bacteria in the presence of additional helper λcI_{857} and again allowed to grow. The c^+ phage type was scored by the colony-sparing unit assay described below.

A recipient K12 ($\lambda cI_{857}\, \mathrm{sus}\, A_{32}B_1$)/F′ trp ($\lambda cI_{857}\, \mathrm{del}_1$) was grown at 30° and infected at low multiplicity with the lysate to be assayed. It was then diluted appropriately and plated for colony-forming units on Tryptone plates. These plates were incubated at 42° for six hours and then at 30° for 24 hours. The number of colonies arising on these plates was a linear function of the concentration of plaque-forming units in the case of $\lambda+$, with an efficiency of 1.2 and a background of 10^2 colony-sparing units per ml.

1) In these experiments, 5-bromouracil was used as the density label. Lambda DNA fully substituted by 5-bromouracil is a poor template both for RNA and for DNA synthesis (Jones and Dove, unpublished).

in either mode of lytic growth.

Mutant t_{27} may be defective in the left-hand promotor. After induction of t_{27}, very little lambda-specific mRNA is synthesized (Guha and Szybalski, personal communication). We shall not go into the possible reasons for its defect in phage yield after induction.

Mutant t_{11} may be defective in the right-hand promotor. After induction of t_{11}, leftward mRNA is made abundantly, but no rightward mRNA is found (Taylor, Hradecna and Szybalski (1967); Cohen and Hurwitz (1967)). We shall discuss ways in which rightward transcription might control replication activity in *cis*.

Mutant t_5 does not revert, maps outside imm_{21} and probably to the left of sus O_8, and shows no transcription defect — it can express P function in complementation (Eisen, *et al.* (1966)). If t_5 is not deficient in rightward transcription, perhaps it is deficient in the replicator for autonomous replication. This suggestion is consistent with the results of physical experiments which show that a preferred origin for lambda DNA replication is in the right arm (Makover (1968 a and b); LePecq and Baldwin (1968); Tomizawa and Ogawa (1968)).

In summary, lambda possesses two primary cistrons for autonomous replication - O and P - whose products may act as an initiating system. These products are phage-specific even among lambdoid phages. Their presumed substrate, the replicator, may be identified by the mutation t_5.

A mutant selection scheme devised by Pereira da Silva has elicited several hundred replication-defective mutants of lambda susN cI_{857} (Eisen, Pereira da Silva and Jacob (1968)). The study of these mutants suggests that the only replication genes outside the imm_{21} segment are O and P[2].

REPLICATION INHIBITION AND REPLICATOR ACTIVATION

Replication inhibition was first discovered by Thomas and Bertani (1964) in phages

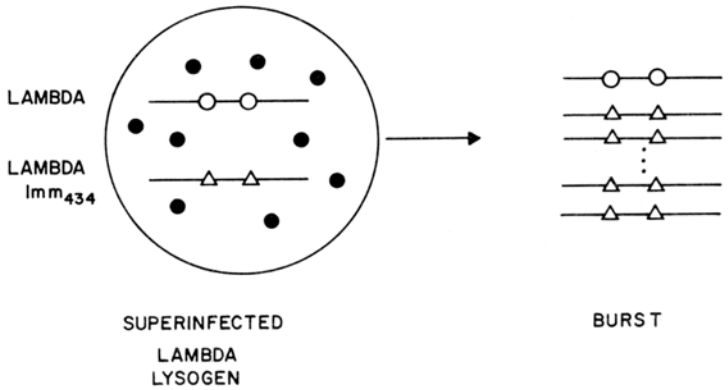

Fig. 2. The Thomas-Bertani experiment.

A lambda lysogen is mixedly infected with lambda and with λimm_{434}. The prophage is not shown. Lambda repressor is indicated ●; it represses lambda, —○—, but not 434, —△—. The burst contains a large yield of λimm_{434}, but no more lambda than the input.

2) Since this selection starts with a susN prophage, it may not elicit mutants defective in genes whose expression is strongly dependent upon N function. The fact that such susN prophages do show some autonomous replication upon induction (Eisen, Pereira da Silva, and Jacob (1968)) suggests that there cannot be such a gene.

lambda and P2. The plan of the Thomas-Bertani experiment is shown in Fig. 2. A lambda lysogen is infected with lambda and with a heteroimmune hybrid such as λimm_{434}. If all the apparatus necessary for autonomous replication can be synthesized by λimm_{434} and can be supplied *in trans,* then repressed lambda should multiply and appear at high yield in the burst. Instead one finds lambda in the burst in numbers no greater than the input. Subsequent experiments employing a density label in the lambda genome have indicated that many of the lambda genomes appearing in the burst have not replicated even once (Ptashne (1965); Green, *et al.* (1967))[3].

Gottesman and Yarmolinsky (1968) have constructed tandem double lysogens carrying an inducible and a non-inducible immunity character. Their experiments suggest that replication inhibition can be bypassed if the immunity-sensitive genome is joined to an actively replicating one. Thus, one presumes that replication inhibition is not directed against the propagation of replication but rather against the initiation step.

Three general kinds of hypothesis can be advanced to explain replication inhibition.

1. The Inhibitor Hypothesis. There is an inhibitor acting upon repressed lambda which directly prevents initiation. A popular form of this hypothesis is that the lambda repressor acts as such an inhibitor; this would require that the lambda replicator be closely linked to a binding site for the lambda repressor.

A more complex form of this hypothesis is that the inhibitor is some other substance, perhaps a bacterial gene product. This requires an additional *proviso* to account for the reversal of replication inhibition upon induction. One suggestion is that the process of transcription would displace such a generalized inhibitor[4].

2. The Non-diffusible Protein Hypothesis. There is a gene whose product is essential for replication, which is repressed by immunity, and which cannot be supplied *in trans* by any heteroimmune or virulent derivative of lambda.

3. The Replicator Activation Hypothesis. The lambda replicator is not active unless there is transcription in its vicinity. Physical models for such activation by transcription will be considered after we discuss the evidence which favors this kind of hypothesis as the basis of replication inhibition in lambda.

We have studied this phenomenon by eliciting mutants which have gained the capacity to multiply in a Thomas-Bertani experiment. Such mutants (which we call replication-inhibition-constitutive or ri^c) can be found simply by an enrichment procedure of repeatedly growing a stock of mutagenized lambda through Thomas-Bertani cycles. Nitrosoguanidine mutagenesis of sufficient intensity to produce 5% clear-plaque mutants produces ri^c mutants at a frequency near 10^{-6}. The particular mutant we have studied most intensively[5] is designated ri^c_{5b}; the wild type is designated ri^+. Deletion mapping with a series of λdbio derivatives and immunity hybrids places 5b to the right of gene cII and to the left of $susO_{29}$, as shown in Fig. 1.

3) In Ptashne's study, only density-labelled prophage lambda was used. One might propose that the prophage would show a replication defect because excision is necessary for replication. In the study of Green and his colleagues, 5-bromouracil was used as the density label. The poor replication of such genomes is evident in this work.

4) We are indebted to Dr. W. Gilbert for this suggestion.

5) Recently, three more ri^c mutants have been isolated and fully studied. All are *cis*-dominant and act as new promotors in the O-P region. Two appear to lie to the left of gene O and one within gene O.

Table 2. Transcription from superinfecting lambda in immune lysogens

Infecting phage	Counts/min RNA hybridized			
	Leftward strand		Rightward strand	
	λcb_2	$\lambda b_2 imm_{21}$	λcb_2	$\lambda b_2 imm_{21}$
None	193	122	0	172
$\lambda+$	346	92	156	177
λri^c_{5b}	435	108	406	578
$\lambda v_1 v_3$	287	359	1005	816
λimm_{434}	18139	14723	5735	4794

The lysogenic recipient W3350 (λind$^-$) was grown in a mineral medium with glycerol as sole source of carbon, supplemented with casamino acids. Superinfecting phages were adsorbed at a multiplicity of 3 in adsorption buffer (0.01 *M* trishyroxymethylaminomethane, pH 7.3, 0.01 *M* $MgSO_4$). Growth was initiated by dilution into growth medium at 37°. RNA was labelled between 3 and 5 minutes after infection by adding tritiated uridine at an activity of 25 microcuries/ml. Cells were lysed in a buffer of 0.15 *M* NaCl, 0.01 *M* trishydroxymethylaminomethane, pH 7.5, 0.02 *M* EDTA, with 0.5% sodium dodecyl sulfate at 100° for 3 minutes. Macaloid was added to 0.2%. RNA was extracted at 60°C three times with hot water-saturated phenol.

Hybridization was done in 0.4 ml of 0.3 *M* NaCl, 0.03 *M* sodium citrate, pH 7, at 62°C for 6 hrs with 2 μg of separated DNA strands from the source indicated. Approximately 3×10^5 counts/min of RNA was used. The filters were loaded with the hybridizing material, treated with ribonuclease, and counted by scintillation spectrometry. Each of the abovs figures was corrected for a blank in which the hybridization was carried out in the absence of DNA. These blank values were in the range 30 connts/min.

We have found that λri^c_{5b} cannot multiply in a lambda lysogen unless a heteroimmune helper phage is also present. It is repressed both for N and O expression, and must therefore possess intact left and right-hand operators. It does express P function in immune lysogens - as judged by its capacity to kill lysogens for λc_{17}susP in the assay of Packman and Sly (1968). Direct measurements of messenger RNA synthesis after infection of a lambda lysogen by ri^c_{5b} confirm this. These results are shown in Table 2.

The expression of ri^c_{5b} is *cis*-dominant: this mutant can multiply in a Thomas-Bertani cycle in the presence of wild type lambda, but it does not help the wild type lambda to multiply. Other mutants studied by other workers also show a *cis*-dominant loss of replication inhibition. The double mutation v_1v_3, which behaves as a constitutive form of the right-hand operator, and the mutation c_{17}, which acts as a new right-hand promotor, both can multiply extensively in a Thomas-Bertani cycle. We have confirmed these observations, as shown in Table 3.

Let us discuss the three hypotheses we have set out to explain replication inhibition. Is ri$^+$ a repressor-binding site? The fact that ri^c_{5b} maps outside of the immunity

Table 3. Multiplication of lambda mutants in lambda lysogens in the presence of helper

Infecting lambda	Total burst size	Lambda in burst	
		Prophage	Infecting
λcI_{60}	31	0.65	0.60
$\lambda cIri^c_{5b}$	81	1.0	35
λc_{17}	35	0.50	10
$\lambda v_1 v_3$	93	1.5	75

The lysogenic recipient W3350 (λind$^-$) was grown, starved, and infected with a mixture of a derivative of lambda (multiplicity 0.1) and a helper λimm_{434}cI (multiplicity 5). After growth and lysis, the burst was titered for total burst size on a sensitive indicator and for lambda in the burst on a lysogen for λimm_{434}. The superinfecting lambda could be distinguished from the prophage by its clear plaque character.

Table 4. Competition between ri^c and ri^+ in the presence of lambda repressor

Host--	594	594 (λ)
Total multiplicity	6.0	7.1
Total burst size	84	110
Input ri^c/ri^+	1.35±0.14	1.35±0.14
Outcome ri^c/ri^+	1.95±0.14	1.98±0.14

A mixture of two phage types, $\lambda imm_{434}c^+ri^c$ and $\lambda imm_{434}cIri^+$, was adsorbed to the host, growth allowed for a single cycle, and the input and outcome titered on an indicator lysogenic for λ. The input and outcome ratios ri^c/ri^+ were calculated from the ratios of turbid to clear plaques. The standard error was estimated by Poisson staistics.

segment suggests that this cannot be true. However, one could imagine that ri^+ is a weak binding site not measured in the binding assay of Ptashne, or that ri^+ acts as a binding site in cooperation with o_r, as if the repressor were "chelated" by the DNA molecule. We have ruled out even this possibility by showing that there is no detectable inhibition of the replication of λimm_{434} ri^+ by the lambda repressor.

A mixture of λimm_{434} $cIri^+$ and λimm_{434} c^+ri^c was grown in the sensitive host 594 and in its lambda lysogen 594 (λ). The ratio of genotypes in the input and bursts was accurately measured by the ratio of clear to turbid plaques. The results of such a competition experiment are shown in Table 4. The ri^c derivative outgrows the wild type phage by a factor of 1.4 in both the sensitive host and the lambda lysogen. No selective inhibition of the ri^+ phage can be detected in the lambda lysogen.

The general inhibitor hypothesis can be tested by asking whether the ri^c allele has *lost* a binding function. This is equivalant to asking, "Which is the active allele, ri^+ or ri^c?" Such a question is answered by a dominance test for genes which act *in trans*; we have been devising a test applicable to the *cis*-acting genes in question in this study (Dove and Davies, work in progress).

If b^+ is the active allele for a binding function, and if this binding site is active in double helical form rather than single strand form, then one would expect that a heteroduplex b^+/b^- would be inactive in binding and would show a phenotype characteristic of the b^- allele. If, instead, the binding occurs to a particular one of the two strands, then the two possible heteroduplex structures b^+/b^- and b^-/b^+ will show different phenotypes.

Preliminary analyses of such heteroduplexes for ri^c/ri^+ indicate they show replication inhibition. In contrast, heteroduplexes $v_1v_3/^+$ lack replication inhibition, consistent with the view that o_r is the active allele for a two-strand binding function.

Thus, we conclude that ri^c_{5b} is the active allele, acting as a new promotor (p'_{5b}) for messenger RNA synthesis from a site within cistron O to the right through cistron P. There is no evidence that ri^+ represents a binding site for any inhibitor. To completely exclude this possibility, however, one needs to have saturated the ways of mutating to ri^c.

Thus, all the evidence we have obtained on the control of replication in lambda, summarized in Table 5, establishes a strict correlation between transcribing activity in the region of the presumed replicator t_5^+ and replication activity. There is no evidence that replication inhibition arises from the action of an inhibitor on the replicator. The correlation between replication activity and transcription, however, does not distinguish

Table 5. Summary of evidence supporting a correlation between transcription activity in the O-P interval and replicative activity

Lambda	Host	Transcription p_l	Transcription p_r	Multiplication in presence of helper
$\lambda+$	Sensitive	←	→	Yes
$\lambda+$	Immune			No
λt_5	Sensitive	(←	→)	No
λt_{11}	Sensitive	←		No
λv_2	Immune	(←	)	No
$\lambda v_1 v_3$	Immune		→	Yes
λc_{17}	Immune	(	→)	Yes
λri^c_{5b}	Immune		→	Yes

Transcription activity is given in parentheses if it is judged only on the basis of complementation. It is given without parentheses if it has been measured directly. In certain cases, it is possible that the levels of transcription measured are underestimates of those occurring *in the presence of helper.*

Table 6. Inability of chloramphenicol to selectively inhibit the multiplication of a genome which enters after the inhibition of protein synthesis

	Removal of chloramphenicol	
	Immediate	Delayed
Total burst size	77	110
Ratio: Second genome/first genome	0.061 ± 0.006	0.063 ± 0.005

Sensitive bacteria C600 were grown in M9 maltose casamino acids thiamine medium, starved, and infected with $\lambda imm_{434}cI$ at a multiplicity of 5. Growth was initiated ($t = 0$) by dilution into growth medium at 37°C. At $t = 10$ min, chloramphenicol (Calbiochem B grade) was added to 200 μg/ml. According to Amati (personal communication) lambda DNA replication continues under these conditions with a rate decreasing with a half-life of 7 minutes. The rate of polymerization of labeled leucine into acidinsoluble material has fallen to 1.5% of normal by 40 seconds. At $t = 11$ min, a second phage, λhc^+, was added at multiplicity 3. Adsorption was 90% complete by $t = 12$ min, and immediate dilution from chloramphenicol was made then; delayed dilution was made at $t = 17$ min. The ratio of the second to the first phage was measured by the ratio of turbid to clear plaque-formers in the burst. The standard error was estimated by Poisson statistics.

between the Non-diffusible Protein Hypothesis and the Replicator Activation Hypothesis.

We can further define the Non-diffusible Protein Hypothesis to state that the gene for this protein must lie to the right of ri^c_{5b} since this mutant acts as a new promotor for transcription to the right from some point within cistron O. Thus, one cannot include segment x, in which there are *cis*-acting replication defectives such as t_{11}. Evidence against the Non-diffusible Protein Hypothesis comes first from the extensive mutant selection of Eisen, Pereira da Silva and Jacob (1968), in which genes O and P have been populated with around 100 independent mutations but no other gene outside of the imm_{21} segment has been discovered[2)]. Secondly, we have asked whether high levels of chloramphenicol can prevent the multiplication of a second phage genome injected into an infective center. This experiment is described in Table 6. We find that, under conditions in which the rate of amino acid polymerization is reduced to 1.5% of normal, an incoming phage genome is at no detectable selective disadvantage in multiplication.

Thus, the Non-diffusible Protein Hypothesis is tenable only if extreme conditions prevail. The most likely source of replication inhibition in lambda is a requirement for replicator activation.

Before discussing this situation, let us remember that there are a number of unre-

2) see page 14.

solved facts involved in reaching this conclusion:

1. What kind of mutation is t_5 — point mutation, deletion, insertion or rearrangement?
2. Does the replicator map to the right of all ri^c mutant sites?
3. Are *all* ri^c mutants new promotors, or are there mutants which represent the loss of a binding site for an inhibitor?
4. What is the transcription pattern for t_5 in a sensitive host and for c_{17} and v_2 in immune hosts (Table 5)?
5. Are all replication-defective mutants outside imm_{21} in cistrons O and P alone (cited by Eisen, Pereira da Silva and Jacob (1968))?
6. Can a phage genome actually replicate when injected into an infective center after the *complete* inhibition of protein synthesis (Table 6)?

In addition to these experimental limitations, let us also remember that the logic leading to this conclusion is only one of correlation. It is clear that the validity of this conclusion can be rigorously established only by establishing the structure of the initiating system — presumably by experiments *in vitro*.

Two general kinds of molecular models can be advanced to account for replicator

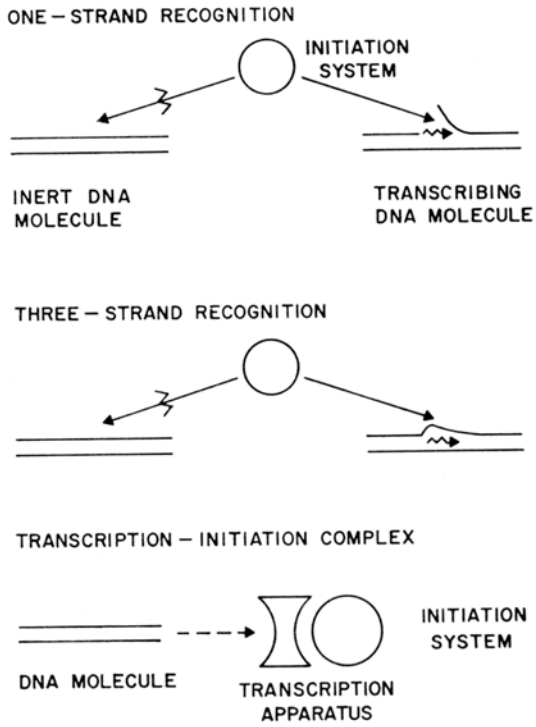

Fig. 3. Models for replicator activation in lambda.

The phage-specific initiating system is symbolized ○. It is presumed to recognize a particular nucleotide sequence represented by t_5^+, but to be able to do this only when there is active transcription of the O-P segment. The one-strand and three-strand recognition models state that during transcription the DNA sequence to be recognized is put into a one-strand or a three-strand state. The transcription-initiation complex model states that the initiating system associates within the cell with the apparatus responsible for the transcription of the O-P segment, Π, and that the lambda DNA molecule can gain access to the initiating system only by passing through this transcription apparatus.

activation by transcription. One states that the structure of DNA changes during transcription, either by transient DNA strand displacement during RNA synthesis, putting the replicator sequence into a single-strand state, or by the formation of a tight three-stranded complex with the nascent RNA chain. In this kind of model, it would be proposed that the initiating system (O and P?) will bind only to such an altered state of the replicator. The other kind of model states that the infected cell is organized in a special way — that the apparatus responsible for initiating DNA replication is bound to the apparatus for transcribing the O-P segment of lambda, and that lambda can gain access to the initiating apparatus only by passing through the O-P transcription apparatus. These models are diagrammed in Fig. 3.

Other systems may achieve "replication inhibition" by other means. In phage P2, a gene A has been identified by both temperature-sensitive and amber mutations (Lindahl, unpublished) and has been implicated in autonomous replication (Lindqvist, unpublished). Mutants in A are not complementable, as if the product of gene A were not diffusible. This may give rise to replication inhibition in phage P2.

What is the significance of replication inhibition in lambda? We have attacked this question by studying the properties of ri^e in contrast to ri^+. We have found that ri^e derivatives are very defective in establishing lysogeny, but that once a lysogen is established it is not detectably unstable with respect to the spontaneous release of phage. Thus, replication inhibition in lambda does not serve to further stabilize the lysogenic state (for example against basal levels of the replication enzymes); rather, it acts during the establishment of lysogeny.

If an ri^e mutant infects a sensitive host under conditions favoring lysogeny, the potential lysogens are not lysed, and phages are not released. Replication inhibition does not control the decision not-to-lyse. However, the potential lysogens are killed if cistrons O and P are functional in a ri^e mutant. (Since O expression is not constitutive in ri^e_{5b}, we presume that the O product relevant to this killing was made under control of p_r rather than p'_{5b}, before immunity was established.)

A second anomaly can be observed during lysogenization by ri^e mutants of lambda. If these mutants are defective in O and/or P, lysogens are formed successfully, but only after a phase of extensive filament formation. Even established lysogens show residual filamentation. This apparent defect in cellular division, caused by the ri^e state and not by the action of the O and P products, depends upon N product but not upon integration. It seems that repressed lambda lacking replication inhibition interferes with the normal course of cellular division. We have no models to suggest in an attempt to understand this phenomenon.

Thus, one role of replication inhibition in lambda seems to be to switch off the replicator as a substrate for initiators made during the phase of autonomous replication preceding lysogenization. One might imagine that analogous processes are necessary for any genome which displays phased initiation in its replication cycle.

In *E. coli* growing at moderate rates, no DNA sequence is replicated twice before every sequence is replicated once (Meselson and Stahl (1958)). This apparent coupling between completion and initiation is strictly observed during balanced growth (Nagata and Meselson (1968)), and may be broken down only transiently by thymine deprivation

(Pritchard and Lark (1964)), or by a shift up in growth rate (Yoshikawa, O'Sullivan, and Sueoka (1964)). One might imagine that the replicators of the daughter chromosomes are activated directly or indirectly by the arrival of a growing point at the terminus.

In the syncytial organism *Physarum polycephalum* it seems that signals controlling nuclear mitosis are freely exchangeable from the cytoplasm (Guttes and Guttes (1964)). However, a G2 nucleus placed in an S-phase cytoplasm cannot be stimulated to enter a replication phase (Guttes and Guttes (1968)). Here, one might imagine that the process of nuclear division activates the daughter replicators, for example by admitting initiators from the cytoplasm or by releasing inhibitors from the nucleus.

In general, one is led by these considerations to ask whether replicators are always available for initiation, or whether they require activation by endogenous processes.

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