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Control of λ Repressor Synthesis

(DNA binding/radioimmune assay/lysogeny versus lysis/*E. coli*)

LOUIS REICHARDT AND A. D. KAISER

Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94305

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ABSTRACT **Direct measurements of the intracellular level of λ repressor have been made by a DNA-filter assay and a radioimmune assay. Transcription of *cI*, the structural gene for repressor, appears to initiate at two different promoters, *prm* and *pre*. Promoter *pre* is activated during the establishment of lysogeny by the action of *cII* and *cIII* proteins at the DNA site *cY*. Phage mutated in *cII*, *cIII*, or *cY* do not make a normal burst of repressor after infection and do not efficiently lysogenize the cell. *Cro* product stops repressor synthesis midway in the infective cycle.**

Promoter *prm* maintains the repressor level in established lysogens. Deletion mapping places it very near the right operator (*Or*). *Prm* is activated by repressor bound to the right operator. In the absence of *cII* or *cIII* protein, repressor synthesis requires active repressor and only proceeds on genomes able to bind repressor at *Or*.

The λ repressor is the only phage protein needed to maintain immunity (1). By binding two operators, *Or* and *Ol* in Fig. 1, this protein directly prevents the transcription of the two early phage operons (2, 3). Consequently, only one operon, that which includes *cI* the structural gene for repressor (2), is transcribed in the presence of active repressor.

Since Eisen *et al.* (4) presented evidence that the synthesis of the λ repressor is regulated, there has appeared strong evidence for a circuit of repressor control in which repressor prevents *cro* gene expression in immune lysogens, and *cro* product prevents repressor synthesis in nonimmune lysogens (5, 6). Other evidence has suggested that *cII*, *cIII*, and *cY*—genes needed for efficient establishment of lysogeny—may also participate in repressor regulation (6–8). Attempting to understand repressor regulation, we have made direct measurements of λ repressor levels—either by measuring its DNA-binding activity (9) or by measuring its combination with antirepressor antibody (10). Among the advantages of the antigen assay are its high specificity, its sensitivity (repressor levels of less than one monomer per cell can be measured), and its utility in detecting repressor unable to bind DNA. The data reveal the existence of two separate pathways of repressor synthesis, one active in the establishment of repression and the other in its maintenance.

MATERIALS AND METHODS

λ Repressor assays

DNA Filter-Binding Assay. This assay measures the ability of repressor, which binds to nitrocellulose filters, to retain [^{32}P]DNA (9). Our procedure has been described by Ordal (11), with one difference: in our experiments, the concentration of chickblood DNA was raised to 132 μg/ml and this DNA was sonicated. In each experiment, λ-specific DNA-binding activity was calculated from the results of parallel

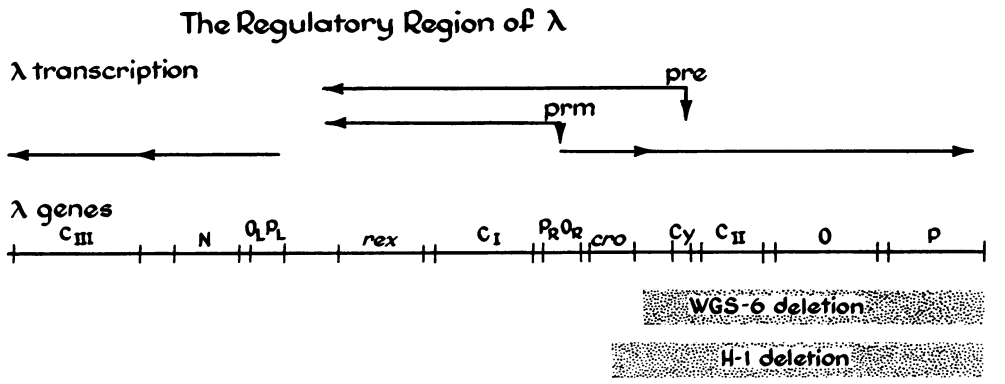

FIG. 1. The regulatory region of λ. This figure details the genes and transcription pattern of 6000 DNA base pairs in the bacteriophage λ. *Thin lines* above the genetic map denote the transcription pattern. *pre* and *prm* mark the postulated locations of two promoters for *cI* transcription. *Pl* and *Pr* are promoters for repressor-sensitive transcription diverging from *cI*. *Ol* and *Or* are the *left* and *right* operators at which repressor binds. *cII* and *cIII* products activate *pre* at the DNA site *cY*. *N* product promotes transcription to the left of *N* and right of *cro* (23, 24). *cro* reduces *N* expression (25) and *cI* expression.

Mutations used in this paper include: v_3, v_1, and vS_{226}, which map in *Or* (2, 11); *sex*, possibly in *Pl*, which reduces leftward transcription of *N* and *cIII* (26), and x_3^- and x_{13}^-, possibly in *Pr*, which abolish rightward transcription of *cro*, *cII*, and *O* (27). The DNA deleted in two prophage strains is denoted below the genetic map.

assays with λ[^{32}P]DNA and λi^{434} [^{32}P]DNA. The protein, which binds λ but not λi^{434} DNA, is sensitive to anti-λ repressor serum (unpublished observations), whether isolated from repressed or derepressed cells.

Radioimmune Assay. When repressor is in excess, unlabeled repressor inhibits the binding of [^{125}I]repressor to anti-λ repressor serum. 5 ng of pure repressor inhibits by 70% the binding of [^{125}I]repressor to antiserum. Since other proteins in extracts do not interfere with the assay (less than 1 pg of repressor antigen/2 μg of protein is found in *Escherichia coli* not lysogenic for λ), the quantity of extract that inhibits binding to the same degree contains 5 ng of repressor.

λ repressor was purified to homogeneity (Reichardt, unpublished). ^{125}I-repressor was prepared by chemical iodination (10). Antiserum to our λ repressor was kindly prepared by Dr. L. Levine of Brandeis University. The buffer for radioimmune assays contains 0.01 M Tris·HCl (pH 7.4), 0.14 M NaCl, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, 0.1 mM dithiothreitol, 5% glycerol, and 0.1% gelatin. Individual assays, in 1.3 ml, contain 0.01 μl of rabbit anti-λ repressor serum, 10,000 cpm of [^{125}I]repressor, and suitable quantities of extract or purified repressor. Tubes are incubated 80 hr at 0°C; 100 μl of 200× diluted normal rabbit serum and 70 μl of sheep antiserum to rabbit globulin are added; after 3 hr, tubes are centrifuged 30 min at 2500 rpm, decanted, dried, and counted in a Nuclear Chicago 1085 gamma counter.

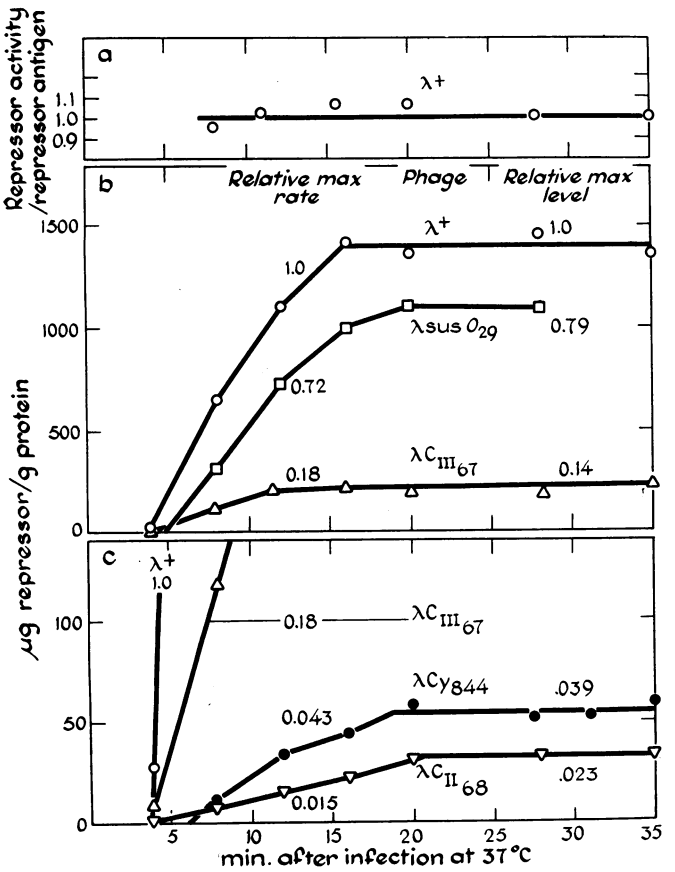

FIG. 2. Rate of repressor synthesis after infection. The rate of repressor appearance at 37°C after infection (protocol 1) with different λ phages are plotted in *b* and *c* on linear scales. The ratio of active repressor to repressor antigen for λ+ infection is plotted in *a*. Phage genotype, the maximum rate, and the maximum level of repressor accumulation (relative to λ+) are listed. λ*sus*O$_{29}$-infected W-3101 (*su*$^-$) at a multiplicity of 8; the other phage infected C600 at a multiplicity of 6. Cell survival was 90–100% after λ+, 7% after λ*cII*, and 21% after λ*cIII* infection.

Phage infection protocols

Protocol 1. This procedure includes a starvation step (60 min at the temperature of growth), favors lysogenization, and has been described by others (8).

Protocol 2. Cells are grown overnight in T broth (1% tryptone–0.5% NaCl), diluted 40-fold into TY broth (T broth + 0.5% yeast extract), and grown to 3 × 10^8 cells/ml. Cells are chilled, centrifuged, and infected in 0.1 volumes of 0.01 M K-phosphate (pH 7.0)–0.01 M $MgCl_2$. 30 min later, the cells are diluted into prewarmed growth medium.

Cells are always chilled to 0°C in a −80°C bath. The detailed procedure for the preparation of extracts will be described elsewhere.

RESULTS

Establishment of repression

The kinetics of repressor synthesis after infection of a sensitive bacterium by λ+, Fig. 2*b*, exhibit three salient features: (*a*) the rate of repressor synthesis during the first 5 min is very low; (*b*) 5–16 min after infection, synthesis is rapid, and (*c*) after 16 min, synthesis is dramatically reduced. The same rate is observed whether repressor antigen or DNA-binding activity is measured (Fig. 2*a*). The kinetic pattern does not reflect phage replication, because λ*sus*O$_{29}$, a mutant unable to replicate (12), shows the same timing of synthesis. The rate of repressor synthesis per cell at 8 min is 80–90 times

TABLE 1. *Repressor levels after infection**

Phage 1	None	Phage 2	
		cY844	cY42
λ$^+$	100		
λ*cY*$_{42}$	2.3	3.9 (0)*	
λ*cY*$_{844}$	3.7		
λ*cII*$_{68}$	0.5	215 (210)	243 (239)
λ*cII*$_{2002}$	1.2		
λ*cIII*$_{67}$	16.3	112 (82)	103 (75)
λ*cIII*$_{am611}$	16.0		
λ*cI*$_{am34}$	0.88†	5.6 (0.9)	6.7 (3.2)
W-3101 (λ$^+$)	6.6		

Cells were grown, infected, and harvested after 30 min growth at 37°C (protocol 1). The multiplicity of each phage was 6. The lysogen W-3101 (λ) was grown at 37°C to 4 × 10^8 cells/ml, and harvested.

* Results are expressed as the percent of the level of repressor accumulated in cells infected by λ+, in this case 1230 μg of repressor per gram of protein. The values in parentheses are the increments of repressor attributable to complementation between phage 1 and phage 2 and are expressed as the percent of the level accumulated in λ+-infected cells. These increments were not calculated from the values in column 1, but instead from a different set of controls infected on the same day.

† The repressor antigen detected after infection with λ*cI*$_{am34}$ has immunological properties suggesting that it is a *cI* gene product. It probably reflects reinitiation of translation beyond *am*34 or weak suppression in W3101. Since phage with amber defects do not plate on W3101, we favor the former possibility.

the rate in a single lysogen (calculation, see *Discussion*); the quantity of repressor accumulated is 10–20 times that found in a single lysogen (Table 1).

Phage bearing mutations in *cII*, *cIII*, or *cY* lysogenize infrequently, but do form stable lysogens (1). Although genes *cII* and *cIII* make diffusible products, amber *cI* mutants are unable to complement *cY* mutants to form lysogens (13). Consequently, *cY* mutations may define a site which regulates *cI* transcription.

Measurements of repressor antigen show that cells infected by *cII*, *cIII*, or *cY* mutants accumulate greatly reduced quantities of repressor after infection (Table 1). The mutations primarily affect the rate, not the pattern, of repressor synthesis (Fig. 2*b* and *c*). As a consequence, *cIII* mutants accumulate 15–30% and *cII* and *cY* mutants only 0.5–4% of the normal quantity of repressor.

Genomes with *cY* mutations can not serve as templates for *cII*, *cIII*-promoted repressor synthesis, even in a mixed infection with a cY^+ phage. In Table 1, sensitive antigen assays show directly that *cY* mutants do not complement each other or an amber *cI* cY^+ mutant. Additional data from the same experiment (Table 1, and unpublished) demonstrate efficient complementation between all other combinations of *cI*, *cII*, *cIII*, and *cY* mutants, with repressor accumulation greater than that after single infection with λ+. Echols and Green (14) have reached similar conclusions about *cII*, *cIII*, and *cY* function.

Why doesn't repressor synthesis continue at a high rate late in infection? From Fig. 3, it is apparent that repressor arrest is due to accumulation of *cro* gene product. Neither a cII^+ cro^- nor a cII^- cro^- phage stops repressor synthesis at the normal time (20–30 min after infection at 30°C), though only the first phage lysogenizes efficiently.

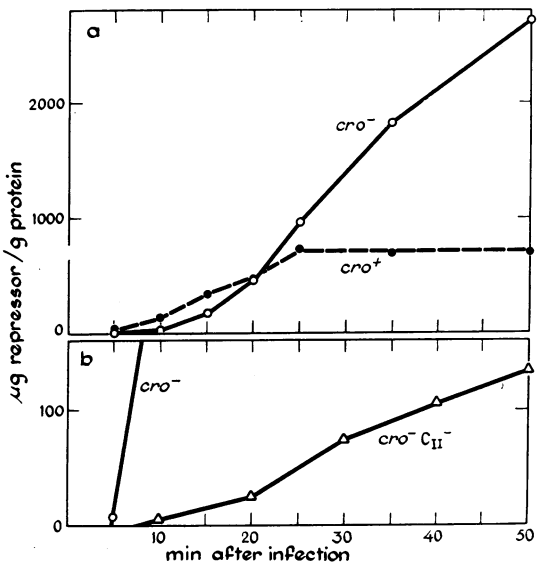

FIG. 3. The effect of *cro* on repressor synthesis. Repressor appearance after infections at 30°C with cro^-, cro^+, and *cII* cro^- phage are plotted on two linear scales. The complete genotypes of the phage were respectively: λ*sex* $cI_{ts857}cro_{27}$, λ*sex* cI_{ts857} (cro^+), and λ*sus*$N_7N_{53}cI_{ts857}$ cro_{27} cII_{2002}. In each case, cells were starved (protocol 1) and infected at a phage multiplicity of 6. LS446 (su_7^+) was the host of the cro^- *cII* infection; C600 was the host for the other infections.

TABLE 2. *Repressor levels in lytic infections*

Phage	Temperature, °C	Shut-off time, min	Assay time, min	μg of antigen / g of protein	% λ+ level*
$\lambda v_3 v_1$	37	20	20	1800	138
$\lambda v_3 vS_{326}$	37	20	20	1600	123
λ+	30	30	30	1200	*100*
λcI_{ts857}	30	30	30	1100	92
λ+	42	10	6	120	*100*
			15	840	*100*
λcI_{ts857}	42	10	6	84	70
			15	460	55

The conditions of infection were those described in the footnote to Table 1.

* Values are expressed as the percent of the repressor level accumulated in λ+-infected cells at the same temperature for the same length of time. The pattern and quantity of repressor accumulation is very temperature dependent (unpublished observations, see column 3).

cII and *cIII* products promote rapid repressor synthesis, even when lysogenization is not possible (Table 2). $\lambda v_3 v_1$ and $\lambda v_3 vS_{326}$, two phages with little affinity for repressor in the right operator *Or* (2, 11), make normal quantities of repressor after infection. Also, a high level of inactive repressor is made at 42°C after infection with λcI_{ts857}. The small difference between cI^+ and cI_{ts857} repressor accumulation at 42°C (30–40%) vanishes at 30°C and reveals the existence of a second pathway of repressor synthesis, dependent on repressor activity.

Maintenance of repression

Repressor is synthesized in a lysogenic bacterium, but the *cII* and *cIII* genes are not expressed (15). Consequently,

TABLE 3. *Repressor levels in lysogens*

	μg of antigen / g of protein	
a) Effect of cII, cIII and cY mutations (37°C)		
C-600 (λ+)	121	
C-600 (λcY_{42})	106	
C-600 (λcII_{123})	105	
C-600 ($\lambda cIII_{67}$)	106	
LS446 (λ^+ $susO_{29}$)	98	
LS446 (λcY_{844} $susO_{29}$)	93	
b) Effect of temperature in cI_{ts} lysogens		
W-3350 (λ+)	81	(30°C)
	82	(42°C)
W-3350 (λ*sus* $N_7N_{53}cI_{ts857}x^-{}_{13}$)	100	(30°C)
	7.7	(42°C)
W-3350 (λ*sus* $N_7N_{53}cI_{ts857}cro_{27}{}^-O^-$)	89	(30°C)
	8.1	(42°C)
c) Effect of cY Deletions (30°C)		
H-1 deletion (cro^-)	86	
H-1 parent (cro^+)	90	
WGS-6 deletion (cro^+)	96	

Cells were grown in TY broth to 4×10^8 cells/ml.

TABLE 4. *Repressor-promoted repressor synthesis*

Phage	cI+ repressor ($\frac{\lambda \text{ DNA-binding units}}{\text{mg of protein}}$)	total repressor ($\frac{\mu\text{g of antigen}}{\text{g of protein}}$)
none	<6	53
$\lambda v_3 v_1 cII_{68}$	<6	53
$\lambda c_{17} cII_{am28}$	60	370
$\lambda c_{17} cII_{am28}$ + $\lambda v_3 v_1 cII_{68}$	45	240

Cells were grown before and after infection in TY broth at 30°C (protocol 2); each phage was added at a multiplicity of 10. Cells were harvested 35 min after infection. The host was the lysogen W-3350 (λsus $N_7 N_{53} cI_{ts857} x_3^- cY_{42}$).

one would not expect repressor synthesis in a lysogen to depend on their action. The data presented in Table 3*a* show that repressor levels in λcII, $\lambda cIII$, and λcY lysogens are, in fact, similar to those in λ^+ lysogens. Thus, the proteins *cII* and *cIII* and their site of action *cY* have no visible role in maintaining the level of repressor.

In the absence of *cII* and *cIII* products, a new pattern of synthesis is observed that requires active repressor and a functional right operator, *Or*. We have measured the rate of repressor synthesis in defective lysogens that are unable to synthesize *cIII* protein after induction. Since the inactive cI_{ts857} repressor made at 42°C retains its antigenicity (Table 2) and is stable (Reichardt, unpublished), the repressor antigen level in a cI_{ts857} lysogen grown at 30 or 42°C will reflect the rate of *cI* gene expression. As shown in Table 3*b*, cI_{ts857} lysogens contain little repressor antigen after prolonged growth at 42°C, although a λcI^+ lysogen has the same antigen level at 30 or 42°C.*

This reduced synthesis seems to result from the absence at 42°C of active repressor, rather than from the appearance of an inhibitor protein. Neither cI_{ts857} prophage makes *cro*, the known inhibitory product.† Moreover, $\lambda sus N_7 N_{53} cI_{ts857} x^-_{13}$ makes no known phage proteins other than repressor and *rex*, both of which are made at 30°C. Thus, it appears that active repressor is required for normal repressor synthesis.

Compatible with this conclusion is a calculation, made along the same lines as that in the *Discussion*, that for 20 min after infection with cII^- or cII^- cro^- mutants, each infecting genome expresses *cI* at less than 20% the rate of *cI* gene expression in an immune lysogen.

If repressor must bind DNA to promote its own synthesis, the right operator (*Or*) is a possible site for such action. To test this possibility, the ability of $\lambda v_3 v_1$ to synthesize repressor was investigated. This phage is unable to bind repressor at the right operator (2). Both antigen and DNA-binding assays are shown in Table 4. The antigen assay detects the cI_{ts857} repressor produced by the prophage; the binding assay does not detect the cI_{ts857} repressor (line 1), and consequently provides a sensitive assay for the repressor made by the cI^+ superinfecting phage. Phage and lysogens are cII^- to prevent *cII–cIII–cY*-dependent repressor synthesis (the polar *x* mutation renders the prophage cII^-). Active repressor accumulates after infection by $\lambda c_{17} cII_{am28}$, which has a normal right operator, but not after infection with the mutant $\lambda v_3 v_1 cII_{68}$. The $v_3 v_1$ defect is recessive, since repressor is made after simultaneous infection with $\lambda v_3 v_1 cII_{68}$ and $\lambda c_{17} cII_{am28}$. The defect appears to be *cis*, as a normal level of cI_{ts857} repressor is maintained in $\lambda v_3 v_1 cII_{68}$-infected cells. Thus, the experiment clearly shows that $\lambda v_3 v_1$ does not serve as a template for repressor-promoted repressor synthesis‡.

Our evidence makes it probable that in the absence of *cII* and *cIII* proteins, repressor promotes *cI* gene transcription (16, 17). Since *cI* is transcribed from right to left (16, 18), *Or* may be part of the promoter, *prm*, from which *cI* transcription originates in a lysogen. The following evidence argues that *prm* is very close to *Or*. Repressor synthesis has been examined in two defective cI_{ts857} prophage (isolated by Spiegelman and by Eisen) deleted from the right prophage end past cY_{42} and in one case into *cro* (Fig. 1). These were originally selected as deletions that retain immunity; consequently, they must either have retained *prm* or fused *cI* to another promoter. The data show that each deletion has, at 30°C, the repressor antigen level characteristic of a λ lysogen (Table 3). Each also has at 42°C a reduced level characteristic of its *cro* genotype (unpublished data†). Therefore, it is likely that *prm* is retained by both deletion strains. Since *cI* is transcribed to the left, *prm* must lie between the right end of *cI* and the middle of *cro*.

DISCUSSION

We propose that *cI* gene transcription originates at two promoters, *prm* (*p*romoter for *r*epressor *m*aintenance) and *pre* (*p*romoter for *r*epressor *e*stablishment), to which proteins must bind to active transcription. Several arguments imply the existence of two distinct promoters: (*a*) Each is defined by a different set of *cis*-acting mutations, *prm* by $v_3 v_1$ and *pre* by *cY*. These two sets cannot belong to the same gene because they are separated by another gene, *cro* (see Fig. 1). (*b*) Different proteins activate the two promoters. Repressor activates *prm*; *cII* and *cIII* proteins activate *pre*. Activation of *prm* does not depend on the site or activators for *pre* and vice versa. (*c*) Repressor synthesis proceeds at different rates from the two promoters.

The following calculation compares the rate of *cI* gene transcription in the presence of repressor with the rate in the presence of *cII* and *cIII* proteins. A single lysogen, doubling every 40 min in growth medium, maintains a repressor level of 80 μg of repressor per gram of protein (Table 1). Therefore, a cell that contains 8.3×10^{10} daltons of protein (the quantity extracted per cell in this experiment) synthesizes each minute 1.45×10^9 daltons of protein and 1.16×10^5 daltons of repressor, or 3.75 repressor monomers of 30,000 daltons each.

On the other hand, 4–16 min after infection by $\lambda sus O_{29}$, 8.3×10^7 daltons of repressor accumulate per cell (8.3×10^{10} daltons of protein), or 2760 repressor monomers in 12

* The *N*, *O*, and *x* mutations prevent cell death and prophage excision in the absence of active repressor.

† Cro^+ cI_{ts857} lysogens make repressor at less than 1% of the normal rate after prolonged growth at 42°C.

‡ The mixed infection demonstrates directly that *cro* product, made constitutively by the phage $\lambda v_3 v_1 cII$, does not prevent repressor synthesis in the period immediately after infection. While *cro* product must do so eventually (28), our observations indicate that 20–30 min elapse after infection at 30°C before this occurs (Fig. 3, Table 2). In addition, we have not detected repressor synthesis after infection of a lysogen with $\lambda v_3 v S_{326} cro_{27}^-$, isolated by Eisen.

min (Fig. 2). Because λ*sus*O_{29} is unable to replicate, the number of *cI* gene copies should remain constant and equal to the multiplicity of phage infection, 8 phage per bacterium. Consequently, 28.8 repressor monomers per minute are made from each genome. Thus, *cI* gene expression can be stimulated 7.5 times more efficiently by *cII* and *cIII* proteins after infection than by repressor itself in a lysogen. In addition, the λ+ infection demonstrates that repressor synthesis in each cell can proceed at 85 times the rate in a single lysogen.

The mechanism by which repressor stimulates *cI* gene transcription might be to stimulate mRNA initiation or to prevent premature termination of a message initiated to the right of *Or*. The latter possibility is rendered unlikely by our observation that *cII*, *cIII*-stimulated repressor synthesis, initiated at *pre*, proceeds in the absence of active repressor or of an effective repressor-binding site (Table 2). The former possibility is supported by the fact that *prm* is located near *Or*, the repressor-binding site.

Since *cII* and *cIII* promote lysogenization of the phage 434, which has a repressor and operators distinct from those of λ (2, 19), it is very unlikely that these proteins bind at *prm* or at the right operator. On the contrary, there is evidence that one or both bind at *cY* or enable another protein to bind *cY*. A DNA site is clearly modified by *cY* mutations as their phenotype is *cis* dominant (Table 1). The *cY* region appears to be the site of *cII* and *cIII* action, since phage bearing a *cY* mutation have both features of *cII* and *cIII* mutants: early lysozyme synthesis (8) and reduced repressor synthesis after infection. Recently, we have detected *cII–cIII–cY*-dependent, leftward transcription of *cro*, with the same samples that supplied the data in Fig. 2 (Eisen, Heinemann, Spiegelman, and Reichardt, unpublished). Consequently, *cY* is a very plausible site for *pre*, although direct evidence that RNA polymerase binds at that site is missing.

The two *cI* gene promoters enable the phage to produce the different quantities of repressor needed for the establishment and maintenance of lysogeny. Clearly, more repressor is needed to establish lysogeny than to maintain it. For example, λ$cIII_{67}$ was able to accumulate 2.5 times the repressor present in a single lysogen (Fig. 2), yet it lysogenized less than 21% of the cells. The two promoters also enable the infecting phage and the integrated prophage to respond differently to sensors of cell metabolism (20).

Features of the repressor regulation system assure that repression is not established before conditions favorable for stable lysogeny have been created. Repressor synthesis depends on gene products from each of the operons that promote lysogeny. The *cIII* gene is transcribed with *int*, a gene essential for integration of the prophage (21); *cII* is transcribed with *O* and *P*, genes required for DNA replication (12). DNA replication promotes lysogeny (22) and repressor synthesis (Reichardt, unpublished). Only after the appearance of repressor is transcription initiated at *prm* to maintain the low repressor level in a lysogen. Consequently, neither *prm* nor *pre* are activated after infection before the other gene products needed to establish stable lysogeny have appeared.

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