Proc. Nat. Acad. Sci. USA
Vol. 69, No. 11, pp. 3156-3160 November 1972

Bidirectional Transcription and the Regulation of Phage λ Repressor Synthesis

(antisense RNA/double-stranded RNA/overlapping operons/*Escherichia coli*)

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Contributed by A. D. Kaiser, August 21, 1972

ABSTRACT **There are two promoters for transcription of gene *cI* in phage λ, the gene that codes for phage repressor. The promoters, called *pre* and *prm*, are located on the distal (*pre*) and proximal (*prm*) sides of gene *cro*, which itself is adjacent to *cI*. Since *cI* and *cro* are transcribed in opposite directions, *cI* transcription initiating at *pre* gives rise to an antisense transcript of *cro*, while *cI* transcription initiating at *prm* does not. *Pre*, active after infection of a sensitive cell, is stimulated by products of phage genes *cII* and *cIII*, and may be located at the site defined by the mutant *cY*. *Prm* is active in an established lysogen. These conclusions are based on measurements of the rates of synthesis of antisense *cro* RNA, *cI* RNA, and repressor protein in infected and lysogenic cells. To measure antisense RNA, an assay based on the formation of nuclease-resistant, double-stranded RNA, specific to the *cro* region, was developed. These results raise the possibility that bidirectional transcription of *cro* has a regulatory function in phage λ.**

The genes and transcription pattern of the regulatory region of phage λ are shown in Fig. 1. Synthesis of the phage *cro* gene product depends on rightward transcription from the promoter *Pr* (1–3), while synthesis of phage repressor requires leftward transcription of *cI* (4, 5). Furthermore, the *cI* gene can be expressed by either of two pathways, one active during the establishment of lysogeny and the other maintaining the repressor concentration in immune lysogens (6). Normal *cI* gene expression after infection requires a DNA site, *cY*, to the right of *cro*, and the *cII* and *cIII* gene products (1, 2, 6–8). Although *cI* expression in a lysogen is independent of these activators and DNA site, it appears to depend on active repressor bound to a DNA site, *Or*, which lies to the left of *cro* (6, 9). If *cY* and *Or* define two promoters for *cI* gene transcription (6), then antisense RNA should be transcribed from the *cro* region during the establishment of lysogeny, but not in an established lysogen. This paper reports a test of this prediction.

Abbreviations: **l** and **r** messenger RNA molecules are synthesized leftward and rightward on the conventionally oriented genetic map of phage λ, and are complementary to the *l* and *r* strands, respectively, of λ DNA.

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MATERIALS AND METHODS

Procedures for growth of cells, pulse-labeling, extraction of RNA, and analysis of labeled RNA by RNA–DNA hybridization have been described (5), as have procedures for phage infection and assay of repressor (6, 9, 10).

Leftward, **l**, and rightward, **r**, messenger RNA molecules have their 5′ → 3′ arrows pointing to the left and to the right on the genetic map as conventionally drawn (Fig. 1). Because, by convention (12, 13), the *l* strand of phage λ DNA is the template for leftward transcription, **l** RNA is complementary to the *l* strand of λ DNA and will hybridize with it. The assay for leftward RNA specific to the *cro* region (**l** *cro* RNA) is described in Fig. 2. RNA concentrations were measured with orcinol (11).

RESULTS

Detection of l *cro* RNA

Since double-stranded RNA is resistant to a mixture of pancreatic and T1 ribonucleases under conditions where single-stranded RNA is completely degraded (14), we suspected that **l** *cro* RNA could be detected as a substance able to anneal with radioactively-labeled **r** *cro* RNA and protect it from nuclease digestion. Our source of labeled **r** *cro* RNA is a prophage deletion strain WGS6 growing in the "S" or nonimmune phase (15). This lysogen, as shown in Fig. 1, retains phage λ DNA only in the *cIII* to *cY* region. In the nonimmune phase, WGS6 makes **r** *cro* and **l** *N* RNA; it accumulates *cro* and *N* gene products; but does not make *cI* RNA or repressor (5, 16).

If labeled RNA from this source is annealed with unlabeled RNA extracted from cells infected with phage λ, a fraction of the labeled **r** *cro* RNA becomes resistant to RNase digestion (Fig. 2). Fig. 3 shows that annealing with increasing amounts of unlabeled RNA results in the protection of an increasing fraction of labeled **r** *cro* RNA. This protection is specific: **r** *cro* RNA is protected; **l** *N* RNA is not protected. If one unit is the amount of protective material that will protect 10% of the labeled **r** *cro* RNA, RNA from cells infected with phage λ may contain as much as 600 units/mg of RNA. Protective activity is not found un uninfected cells (<3 units/mg of RNA).

Prior treatment with DNase does not destroy this substance, but RNase destroys more than 99% of the protective activity. Formation of the nuclease-resistant complexes requires prolonged incubation of the mixture at 70°. In the

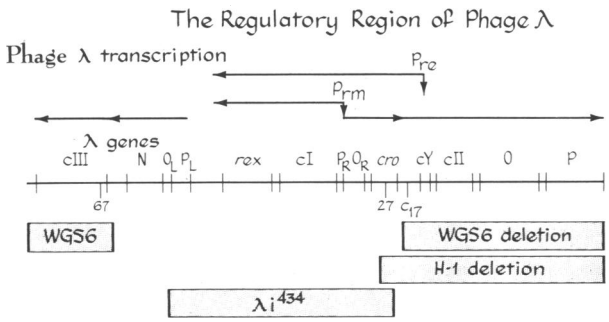

FIG. 1. Genes and transcription pattern of a 6000-base-pair segment of the phage λ genome. DNA segments deleted in two prophage strains (WGS6 and H-1) and one substitution phage ($λi^{434}$) are denoted below by *bars*.

absence of annealing, RNA from cells infected with phage λ retains less than 2% of its protective capacity. The complexes, once formed, melt in 0.15 M NaCl between 85° and 90° (data not presented), the same range in which RNA–RNA duplexes melt (14). Protective substance can be purified by prior hybridization to *l* strands of phage λ DNA, but not to *r* strands. For these reasons, we conclude that the nuclease-resistant complexes are duplexes of unlabeled **l** *cro* RNA and labeled **r** *cro* RNA.

Though different extracts appear to contain different amounts of protective RNA, as shown in Fig. 3, at low concentrations of unlabeled RNA, the quantity protected is proportional to the amount of RNA added. We therefore take the initial slope as a measure of the amount of **l** *cro* RNA in an extract.

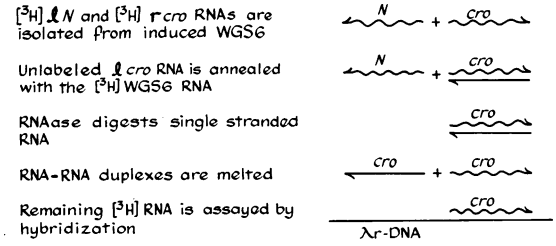

FIG. 2. The assay for **l** *cro* RNA. Strain WGS6 (Fig. 1) was labeled from 5 to 9 min after induction at 42° with [³H]uridine (20 Ci/mmol), and the RNA was extracted (5). Unlabeled RNA from another source was annealed with the WGS6 [³H]RNA for 3 hr at 70° in 0.3 M NaCl–0.03 M Na_3 citrate to form RNA–RNA duplexes, then digested for 3 hr at 37° with a mixture of pancreatic RNase (10 μg/ml), T1 RNase (1 μg/ml), and pancreatic DNase (10 μg/ml) in 220 mM NaCl–10 mM Na_3-citrate–23 mM Tris·HCl (pH 7.5)–10 mM $MgCl_2$–8 mM EDTA. Nucleases were inactivated by addition of an equal volume of 0.3 M sodium iodoacetate in 0.2 M potassium acetate (pH 5.2)–1.8 M NaCl, incubation at 55° for 40 min, and extraction with phenol (4). The RNA duplexes were precipitated with 2 volumes of ethanol at −20°, dissolved in 10 mM potassium acetate (pH 5.2)–1 mM EDTA, melted by heating at 95° for 15 min, quenched in ice water, and hybridized to separate strands of phage λ DNA (5). The fraction of the input [³H]l *N* or [³H]r *cro* RNA that has been converted by annealing to nuclease-resistant form was calculated by comparison to a control sample, which had been subjected to the same manipulations without treatment with nuclease.

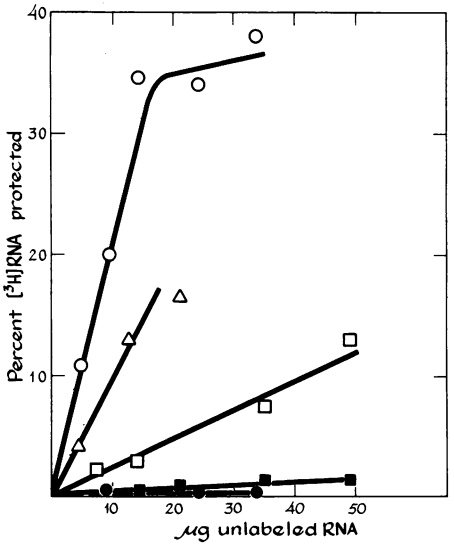

FIG. 3. Protection of [³H]r *cro* and [³H]l *N* RNAs by unlabeled RNA from cells infected with phage λ. Unlabeled RNA samples, which originate from C600 bacteria infected with phage λ, were harvested after growth at 37° for 8 min (○,●), 20 min (△), or 36 min (□,■). *Open symbols*, *cro* RNA; *closed symbols*, *N* RNA.

As shown in Fig. 3, the fraction of **r** *cro* RNA protected increases to a maximum value of 30–40%. Results of several experiments support the hypothesis that this limit is set by the reformation of RNA–RNA duplexes during the final hybridization to the *r* strands of phage λ DNA. Since RNA–RNA duplexes are not retained on nitrocellulose filters, radioactive RNA in such duplexes would be lost in the procedure outlined in Fig. 2. In one experiment, reformation of RNA–RNA duplexes was slowed by lowering the annealing temperature from 67° to 63° (14). This increased the maximal amount of **r** *cro* RNA protection to 63%. In another experiment, **r** *cro* RNA was first hybridized to *r* strands of phage λ DNA, dissociated, then annealed to **l** *cro* RNA. After treat-

TABLE 1. *Effect of mutations in repressor regulatory genes on the amount of* **l** *cro RNA and* **l** *cI-rex RNA*

		Experiment 1		Experiment 2	
Phage	Cells	**l** *cro* RNA	Repressor rate	**l** *cI-rex* RNA	Repressor rate
$λ^+$	C600	100	100	100	100
$λcIII_{67}$	C600	16	18	7.6	—
$λcII_{68}$	C600	2.0	1.5	0.54	—
$λcY_{42}$	C600	2.4	2.6	2.1	—
—	C600($λcY_{42}$)	—	1.0	1.6	1.6

Values are expressed relative to corresponding infections with $λ^+$. $λ^+$ synthesized 380 repressor monomers per cell-min and 238 units of **l** *cro* RNA per mg of RNA (Exp. 1), and 234 repressor monomers per cell-min and 11.4 cpm *cI-rex* RNA per 10,000 cpm total RNA (Exp. 2). The amount of **l** *cro* RNA was measured 8 min after infection; the rate of *cI-rex* RNA synthesis was determined by pulsing the cells with [³H]uridine 8–9.5 min after infection; the rate of repressor synthesis is averaged between 4 and 12 min after infection at 37°.

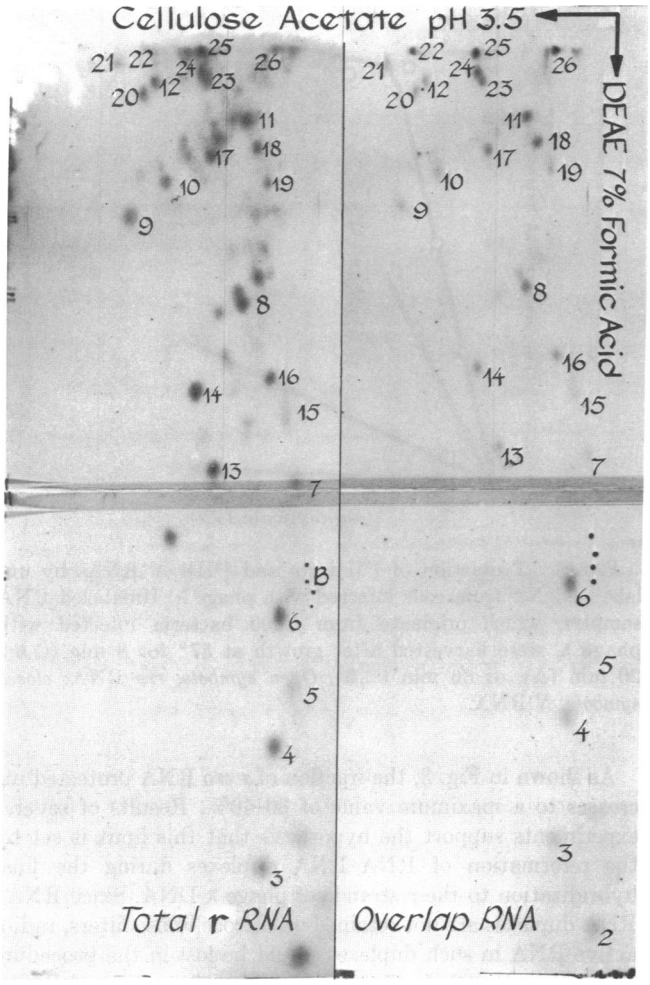

FIG. 4. Two-dimensional electrophoresis of T1 ribonuclease digests of overlap RNA and total rightward phage λ RNA isolated from strain WGS6. Bacteria were pulse labeled with [^{32}P]orthophosphate after thermal induction. RNA was purified as described (5) with the additional steps of extraction with $CHCl_3$–CH_3OH and filtration through nitrocellulose membranes. Labeled RNA was hybridized to phage λ *r* DNA strands, and rightward RNA was recovered (5). One portion was further purified by a second cycle of annealing to *r* DNA strands, yielding "rightward (r) RNA." The remainder was annealed with unlabeled RNA extracted from cells infected with phage λ, and the fraction that formed nuclease-resistant complexes was isolated as described in the legend to Fig. 3, yielding "overlap RNA." Samples were digested with T1 RNase, and the resulting oligonucleotides were fractionated by two-dimensional ionophoresis (17). Oligonucleotides 1–12 are present in digests of total rightward RNA isolated from a strain with the H-1 deletion (Fig. 1). Oligonucleotide 1, Gp, is not shown.

ment with nuclease, 92% of the ^{3}H remained acid-insoluble, indicating that, under the proper conditions, virtually all the r *cro* RNA can be annealed to 1 *cro* RNA.

This conclusion was confirmed in an independent way. ^{32}P-labeled r *cro* RNA was purified by hybridization to *r* strands of phage λ DNA. The nuclease-resistant complexes formed between this labeled RNA and unlabeled RNA from infected cells was also purified. Both preparations were digested with T1 RNase, which specifically cleaves at G residues, and the resulting oligonucleotides were separated by two-dimensional ionophoresis (17). Most, if not all, of the oligonucleotides derived from r *cro* RNA purified by hybridization to DNA are also seen in the digest of r *cro* RNA purified by annealing to 1 *cro* RNA (Fig. 4), indicating again that the entire sequence can be protected by overlapping RNA.

Regulation of 1 *cro* RNA synthesis

After infection of sensitive bacteria, the amount of 1 *cro* RNA per cell first increases then decays (Fig. 5). The rate of repressor synthesis in these bacteria follows the same time course, implying that 1 *cro* RNA is the promoter-proximal part of the 1 *cI* messenger RNA synthesized immediately after infection. Further support for this view is derived from measurements of the amount of 1 *cro* RNA made after infection by *cII*, *cIII*, or *cY* mutants. These mutants synthesize repressor at greatly reduced initial rates (6). As shown by the data reported in Table 1, reduced rates of repesssor synthesis are correlated with reduced amounts of 1 *cro* RNA. We conclude that *cII* and *cIII* gene products and the *cY* DNA site lead to repressor synthesis by stimulating joint leftward transcription of *cro*, *cI*, and *rex*.

Repressor synthesis in an established lysogenic bacterium is independent of *cII* and *cIII* gene products and the *cY* site (6). Infection of an immune lysogen can increase the rate of repressor synthesis apparently by virtue of the increased number of copies of the *cI* gene per cell. As shown in Table 2, the rate of synthesis of repressor in a lysogenic bacterium, infected or not, still reflects the amount of *cI* gene transcription. However, there is no longer a correlation with 1 *cro* RNA. Little, if any, 1 *cro* RNA is made either in the infected or uninfected lysogen. The amount of 1 *cro* RNA (0.9) recorded for the infected lysogen is less than 10% the amount required to explain the observed rate of repressor synthesis under these conditions, and may represent nonspecific protection of r *cro* RNA by the high concentration of RNA present in these samples.

Can the failure to observe 1 *cro* RNA be explained by the presence of an inhibitor in the RNA extracts? To examine this possibility RNA from bacteria (strain C600) infected with phage λ that, as shown above, contains 1 *cro* RNA, was assayed for its content of 1 *cro* RNA in the presence of a 30-fold excess of RNA from C600 bacteria infected with phage λcY_{42} or with phage λ cII_{68} or RNA from uninfected C600 bacteria.

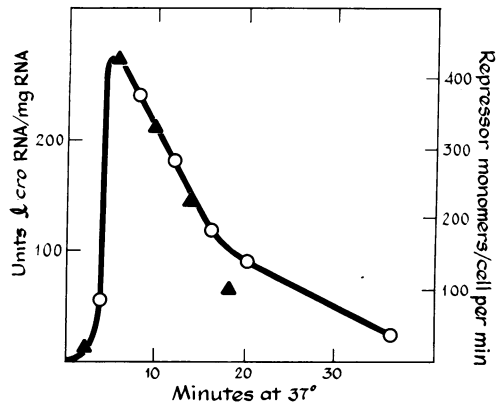

FIG. 5. Time-course of 1 *cro* RNA (○) and phage λ repressor (▲) synthesis. Strain C600 was infected with phage λ at a multiplicity of 6 and diluted with growth medium (5) at 37°. Samples were collected at various times thereafter and assayed for repressor antigen (6) and 1 *cro* RNA (Fig. 1).

In all three experiments, 60–80% of the 1 *cro* RNA was detected by the hybridization assay. Thus, although a large excess of RNA may partially inhibit hybridization, there is no evidence for a specific inhibitor.

Since 1 *cro* RNA would thus have been detectable were it present in extracts of infected lysogenic bacteria, its absence means that such cells fail to produce it. *cI* transcription observed in these bacteria must therefore start at a site to the left of the *cro* gene.

Evidently, there are two promoters for *cI* transcription. It follows from the data presented in Tables 1 and 2 that both types of *cI* messenger RNA are translated by ribosomes with about equal efficiency.

DISCUSSION

When a sensitive bacterium is infected by phage λ there is leftward transcription of the *cro*, *cI*, and *rex* genes that is dependent on the *cII* and *cIII* gene products and the *cY* DNA site. This transcription is "sense" for *cI* and *rex*, but "antisense" for *cro*. The rate of repressor synthesis is proportional to the amount of 1 *cI-rex* RNA and that of 1 *cro* RNA under these conditions. Synthesis of *rex* gene product after infection of a sensitive bacterium has, like repressor, been shown to require a functional *cY* site (18). Thus, after infection of a sensitive bacterium, leftward transcription of *cro*, *cI*, and *rex* appears to be coordinate. Since all of the nucleotide sequences in the "sense" rightward transcript of the *cro* gene are protected by hybridization to the antisense leftward *cro* RNA, a promoter for repressor-gene transcription must lie to the right of the *cro* gene**. Since this promoter is active during the establishment of lysogeny it is called *Pre*, *p*romoter for *r*epressor *e*stablishment. Mutations of the *cY* class lie immediately to the right of *cro*, and they are *cis* dominant (2, 6, 7). *cY* is therefore a plausible location for *Pre*.

By contrast, the experiments reported in Table 2 show that transcription of *cI* and *rex* in an established lysogen starts to the left of the *cro* gene. Leftward *cro* RNA is not observed under these conditions. Repressor synthesis in a lysogen does not depend on *cII*, *cIII*, or *cY;* moreover, normal rates of repressor synthesis are found in a lysogen deleted for the right half of *cro*, *cY*, *cII*, and genes to the right (6, 9). Also in a lysogen, *rex* is regulated coordinately with repressor (1, 15, 19). Thus, there must exist a second promoter for the *cI* and *rex* genes that is active in an established lysogen. This promoter, called *Prm* (*p*romoter for *r*epressor *m*aintenance), most probably lies between *cro* and *cI* genes. Normal repressor synthesis from this promoter requires active repressor bound to the right phage λ operator, *Or*, even in the absence of *cro* gene product (6, 9).

The messenger RNAs transcribed from *Pre* and *Prm* are translated with about equal efficiency. Therefore, the 5- to 10-fold greater rate of repressor synthesis during the establishment of lysogeny compared with that in an established lysogen (6, 8) is due to a greater number of transcripts from *Pre* in the presence of *cII* and *cIII* gene products than from *Prm* in the presence of repressor. This could result from a greater frequency of messenger initiation or a lesser frequency of messenger degradation. In any case, the two *cI* gene promoters enable the phage to synthesize repressor at the different rates required for the establishment and maintenance of lysogeny. They also enable proteins and cell metabolites, including possibly cyclic AMP (20, 21), to regulate independently repressor synthesis in the infected cell and lysogen.

Granting that two different promoters are required to permit two different ranges of rates of repressor synthesis, what significance lies in the fact that one of them lies to the right of *cro*, thus forcing its transcription in two directions? One possibly is that since, in phage λ DNA, sites appear to be adjacent to the structural genes for the proteins that act at those sites (22), *Pre* must be adjacent to *cII*, its major activator. Another possibility is that competition between opposing operons may regulate the growth cycle of phage λ (1). In particular, *cII* and *cIII* gene products, known to delay rightward transcription of genes *Q* and *R* (10), may do so because polymerase complexes initiating at *Pre* interfere with rightward transcription from *Pr*. Finally, it is possible that the formation of double-stranded *cro* RNA *in vivo* may reduce synthesis of the *cro* gene product in infections leading to lysogeny.

TABLE 2. *Repressor synthesis and leftward transcription of cro, cI, and rex in a lysogen and nonlysogen*

Phage	Bacteria	1 *cro* RNA	1 *cI-rex* RNA	Repressor rate
λ+	C600	100	100	100
λ*cY*42	C600(λ*cY*42)	0.9	10.8	8.3
—	C600(λ*cY*42)	<0.5	1.65	1.6
—	C600	<0.5	—	—

Values are expressed relative to a λ+ infection, in which 630 units of 1 *cro* RNA per mg of RNA, 11.4 cpm *cI-rex* RNA per 10,000 cpm total RNA, and 238 repressor monomers per cell-min were seen. In the λ+ infection, 1 *cro* RNA was measured at 8 min; *cI-rex* RNA synthesis was measured from 8 to 9.5 min; and the rate of repressor synthesis was averaged between 4 and 12 min after infection. In the λ*cY*42 infection of C600(λ*cY*42), 1 *cro* RNA was measured at 16 min; *cI-rex* RNA synthesis was measured from 16 to 17.5 min; and the rate of repressor synthesis is the average observed between 0 and 40 min after infection. Repressor synthesis proceeds linearly in this type of infection (9). A *cY* mutation was included in the prophage and superinfecting phage to ensure that transient titration of previously existing repressor by superinfecting phage would not activate the *cII–cIII–cY*-dependent promoter *Pre*.

This work was supported by NIH Grants AI08573, GM18075, and AI04509, and NSF Grant GB7287, as well as a Dernam Junior Fellowship (J-157) of the American Cancer Society, California Division to W. G. S.

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** This assumes that the 12 *cro* RNA labeled *in vivo* represents a transcript of the entire *cro* gene.

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