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Biochemistry

Promoter for the establishment of repressor synthesis in bacteriophage λ

(*in vivo* initiation/positive regulation/convergent transcription/cloning of λ *cro-y-cII* fragment)

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ABSTRACT **Transcription of the λ repressor gene (*cI*) is positively regulated by the phage-encoded proteins cII and cIII. We have isolated and characterized the 5′-terminal region of this RNA and shown that it originates at a promoter (p_E) located between genes *cro* and *cII*. The DNA sequence of this promoter shows little homology to other known promoters. Initiation of transcription from p_E is abolished by the *cis*-dominant mutations *cY*; these mutations alter the "−10" and "−35" regions of the promoter. We propose that the "−35" region is the site of activation of p_E, possibly via the direct interaction of protein cII.**

When bacteriophage λ infects *Escherichia coli*, either of two possible pathways of development may ensue. The phage can reproduce, forming progeny particles that are released upon lysis of the host cell (lytic pathway). Alternatively, the phage may enter a lysogenic state, in which the viral genome becomes integrated into the bacterial chromosome and most phage functions are repressed. Crucial for the establishment of lysogeny is the presence of the λ integrase (int) and repressor (cI) proteins. Synthesis of both proteins is positively regulated by the phage gene products cII and cIII (for reviews see refs. 1–4).

Transcription of the repressor gene is known to be controlled by two distinct promoters. The maintenance promoter, p_M, functions primarily in the lysogenic state (5–7). This promoter lies immediately adjacent to the coding region of gene *cI* and is positively regulated by the cI repressor (8). The other promoter, p_E, is required to establish synthesis of repressor after infection (5–7). In contrast to p_M, this promoter is located quite far from the *cI* gene, somewhere to the right of gene *cro* (ref. 9; see Fig. 1). Transcription of gene *cI* from promoter p_E requires the phage cII/cIII functions. It has been suggested that the cIII protein acts indirectly on the establishment of repressor synthesis, because mutations in both the host [*hfl*$^-$ (14)] and the phage *cII* gene [*can*$^-$ (15)] bypass the requirement for cIII function. Moreover, a regulatory site (*cY*, defined by mutations) is important for cII/cIII-controlled transcription of gene *cI* (5–7). This site lies near the beginning of gene *cII* (16, 17). Although it has been postulated that the *cY* mutations inactivate the p_E promoter (5–7, 18), other plausible hypotheses have been suggested. These mutations could create a new site for termination of transcription or a site susceptible to ribonuclease action (19, 20).

In this paper we show that a λ transcript dependent on the cII/cIII functions is initiated between genes *cro* and *cII*, at the *cY* site, and we conclude that the λ cII/cIII products activate initiation at this promoter.

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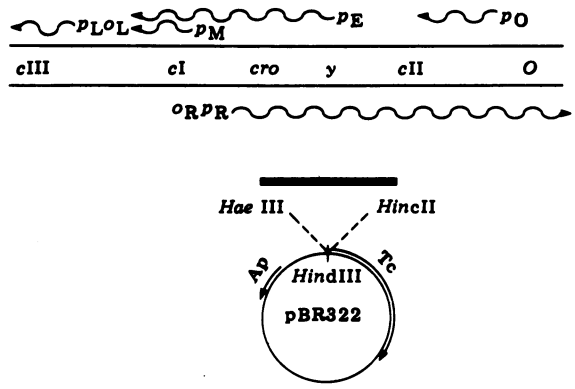

FIG. 1. Partial genetic map and transcription pattern of the λ genome. Directions of transcription are indicated by wavy arrows. $o_L p_L$ and $o_R p_R$ represent the left and right operator/promoter regions (8). Genes *cro*, *cII*, and *O* are expressed from the p_R transcript. p_O is the promoter for the 4S (*oop*) RNA (10–12). p_M is the maintenance promoter for *cI* expression (active in a lysogen) (8, 13). p_E, the promoter for the establishment of repressor synthesis, is determined in this paper. The 398-bp DNA restriction fragment used for hybridization (see text) is depicted below the map. This fragment was inserted into the *Hin*dIII site of the vector plasmid pBR322. Ap and Tc designate the regions of the plasmid conferring resistance to ampicillin and tetracycline, respectively.

MATERIALS AND METHODS

Cloning of a λ DNA Segment. A 398-base-pair (bp) *Hae* III-*Hin*cII DNA restriction endonuclease fragment that has been shown to contain the *cro-y-cII* region of λ was obtained in purified form by polyacrylamide gel electrophoresis. *Hin*dIII linkers (Collaborative Research, Waltham, MA) were ligated to the fragment according to published procedures (21). The modified fragment was then ligated to pBR322 plasmid DNA that had been treated with *Hin*dIII endonuclease and bacterial alkaline phosphatase (Sigma) (21). The ligated DNA was used to transform *E. coli* K-12 strain C600. Plasmid DNA isolated from some of the resulting transformants was tested for the presence of a single fragment of approximately 400 bp at the *Hin*dIII site of the vector. The desired recombinant plasmid was designated pCY398 and its structure was confirmed by detailed restriction analysis (not shown).

Isolation of λ mRNA Complementary to the Cloned λ DNA Segment. ^{32}P-Labeled RNA was prepared as described by Court *et al.* (22). The general procedures for RNA·DNA filter hybridization described below were derived from those of Bøvre and Szybalski (23). Each DNA filter (8 mm in diameter, Schleicher & Schuell) contained approximately 10 μg of plasmid DNA. To facilitate the isolation of a mRNA with an

Abbreviation: bp, base pair.

intact 5′ terminus, some conditions were modified. Hybridization was at 67°C for 6 instead of 18 hr. The filter-bound hybrids were treated with ribonuclease for 20 min at 0°C instead of room temperature, and after careful washing, the filters were incubated in distilled water at 90°C for 3 instead of 10 min. In all cases the eluate was extracted with phenol and the RNA was precipitated with ethanol. Except for giving slightly poorer yields of hybridized RNA, this method did not change the selective hybridization of RNA.

Characterization of the RNA. The [^{32}P]RNA fragments were digested with T1 or pancreatic ribonuclease and the resulting oligonucleotides were fractionated in two dimensions by the standard fingerprinting procedure (ref. 24; see also legend to Fig. 2). For determination of the 5′-terminal nucleotide, the [^{32}P]RNA fragments were digested with nuclease P1 (25), which degrades RNA to mononucleoside 5′-phosphates (26). The digestion products were resolved in two dimensions by thin-layer chromatography on polyethyleneimine plates. In the first dimension, a stepwise separation was carried out with 0.5, 2, and 4 M sodium formate (step formate) at pH 3.4, and for the second dimension, KH_2PO_4 (0.75 M) was used (27).

RESULTS

Isolation and characterization of transcripts from the *cro-y-cII* region of λ

RNA synthesis proceeds in both directions through the *cro* gene of phage λ. Rightward transcription has been well characterized; it is initiated from the p_R promoter and extends through genes *cro* and *cII* (28, 22). Leftward transcription has been detected (9), but its site of initiation has not been demonstrated. Our objective was to analyze the transcription occurring within the *cro-y-cII* region of phage λ during a normal infection, to determine whether leftward repressor gene transcription initiates within this region or traverses it.

For this purpose, a DNA fragment of 398 bp containing the distal half of the *cro* gene (29), the 120-bp intercistronic *y* region between *cro* and *cII* (30), and the proximal two-thirds of the *cII* gene (31), was isolated and inserted into pBR322. DNA from the resulting recombinant plasmid (pCY398) was then used to hybridize the leftward and rightward transcripts made from this region after phage infection. The RNA was labeled by exposing cells to [^{32}P]orthophosphate between 8 and 9.5 min after phage infection. The time at which labeling was started is optimal for expression of the repressor gene (9, 32). The short 1.5-min labeling period was chosen to minimize possible processing, degradation, or both, of [^{32}P]RNA due to ribonuclease activity. Further details about the isolation of RNA from the *cro-y-cII* region are given in *Materials and Methods*. The purified RNA was characterized by digestion with T1 ribonuclease and separation of the resulting oligonucleotides by standard two-dimensional fingerprinting techniques. We obtained T1 fingerprints of RNAs isolated identically from cells infected with different λ derivatives.

Fig. 2 shows T1 fingerprints of RNAs isolated after infections with λ cII^- (*A*), λ cY^- (*B*), or λ cII^+ cY^+ (*C*). Each characteristic T1 oligonucleotide containing seven or more nucleotides was further characterized by digestion with pancreatic ribonuclease followed by electrophoresis in one dimension on DEAE-paper at pH 3.5 or 1.7. By comparing the results of these analyses with a list of potential T1 oligonucleotides predicted from the DNA sequence of the 398-bp fragment (see legend to Fig. 2), each T1 oligonucleotide could be identified and assigned unambiguously to one or the other strand of the DNA fragment.

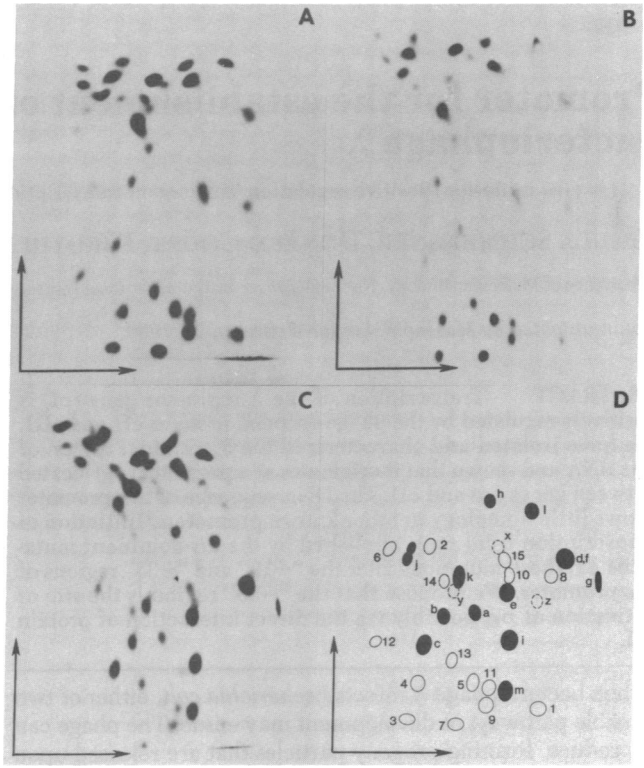

FIG. 2. Two-dimensional fingerprints of ribonuclease T1 oligonucleotide products derived from RNA transcribed in the *cro-y-cII* region of phage λ: autoradiographs (*A–C*) and a composite schematic representation (*D*). Horizontal dimension: electrophoresis on Cellogel strips at pH 3.5. Vertical dimension: chromatography on thin-layer plates of DEAE-cellulose developed with 30-min-hydrolyzed homochromatography C buffer. The RNAs were isolated after infections of *E. coli* K-12 SA500 (*his, rpsL*) with $\lambda cI_{14}cII_{28}cIII_{611}$ (*A*), $\lambda cI_{14}cY_{42}$ (*B*), and λcI_{14} (*C*) (33). Characteristic oligonucleotides of each fingerprint were analyzed by secondary digestion with pancreatic ribonuclease and correlated with the DNA sequence (30). These oligonucleotides are combined in the schematic drawing of *D*. Open circles numbered 1 through 15 represent oligonucleotides derived from p_R-promoted, rightward RNA in the order of their occurrence and are seen in all three fingerprints. They correlate with the predicted oligonucleotides between residue positions 129 and 526 as follows: 1, $AU_7AACUAUA_3CG$; 2, C_3UUC_3G; 3, $UAACA_7CAACAG$; 4, $CAUA_3UAAC_4G$; 5, CUCUUACACAUUCCAG; 6, A_5G; 7, $CAUCA_3UUA_3CCACACCUAUG$; 8, CAU_3AU_3G; 9, CAUACAUUCAAUCAAUUG; 10, UUAUCUAAG; 11, $A_3UACUUACAUAUG$; 12, CA_3CA_3CG; 13, $CUUAACA_4UCG$; 14, $AUUCCA_3G$; 15, UUCUCAAUG. Filled circles marked a through m correspond to oligonucleotides derived from leftward RNA in the order of their occurrence. These oligonucleotides are found exclusively after infection with λ cII^+ cY^+ (*C*) and have the following sequences: a, AUAACAAUUG; b, CA_3UA_3UG; c, CAUACACCAUAG; d, U_3AAU_3G; e, C_3U_5CAG; f, $UUAU_3AUG$; g, U_7G; h, UUACUCG; i, $CU_3ACCUCUUCCG$; j, $CAUA_3CG$; k, CUUCCAUCAG; l, U_3AUAG; m, UUA_7UCU_3CG. Oligonucleotides a–m correlate with the DNA sequence between residue positions 319 and 129. Oligonucleotides z, y, and x (indicated by broken lines) and oligonucleotides w, v, and u (not shown) are predicted from the l-strand DNA sequence between residues 320 and 526. The positions of these oligonucleotides on the T1 fingerprint were determined by the analysis of an *in vitro* end-to-end transcript of the *cro-y-cII* λ DNA fragment (data not shown). None of the oligonucleotides z–u appears on the T1 fingerprint in *C*. Their sequences are: z, $UAU_3CCUUAG$; y, AACCAUAUG; x, UCUUCUCAG; w, ACUUAUCAACG; v, $UC_3UCUUCCACCUG$; u, $AC_6AUUCAAG$. A fingerprint similar to the one shown in *B* was obtained with RNA from an infection with $\lambda cI_{14}cY_{2001}$ (not shown).

Examination of the T1 oligonucleotide fingerprints obtained with RNA isolated from either the λ *cII*⁻ or λ *cY*⁻ infection (Fig. 2 *A* and *B*, respectively) indicates that the oligonucleotides (1–15, in *D*) derive exclusively from the p_R-initiated rightward transcript of the entire *cro-y-cII* region. This same oligonucleotide pattern has been observed previously in studies of both the *in vitro* and *in vivo* rightward transcription products of this region (22). None of the oligonucleotides for the leftward transcript of this region was detected.

In contrast, the T1 oligonucleotide fingerprint obtained by using RNA isolated from an infection with λ *cII*⁺ *cY*⁺ (*C*) is markedly different. Although the oligonucleotides characteristic of the p_R-promoted rightward transcript are still present, albeit in reduced amounts, a new set of prominent oligonucleotides is observed (a–m, in *D*). These oligonucleotides must derive from a leftward RNA transcript of the *cro-y-cII* region. Alignment of each of these with the known nucleotide sequence clearly indicates that only a portion of the 398-bp region is represented. The presence of T1 oligonucleotides a–m indicates that the sequence between positions 320 and 129* is being transcribed into RNA. None of the oligonucleotides predicted by the sequence preceding residue position 320 (i.e., oligonucleotides z–u between positions 320 and 526; see legend to Fig. 2) is detectable on the fingerprint. Thus it appears that this region is not transcribed into RNA during the labeling period. Furthermore, the presence of the T1 oligonucleotide a, AUAACAAUUG, on the fingerprint, and the absence of the immediately adjacent oligonucleotide z, UAUUUCCUUAG, indicates that the 5′ end of the leftward RNA transcript must occur within the sequence defined by oligonucleotide z, somewhere between nucleotide position 330 and 320.

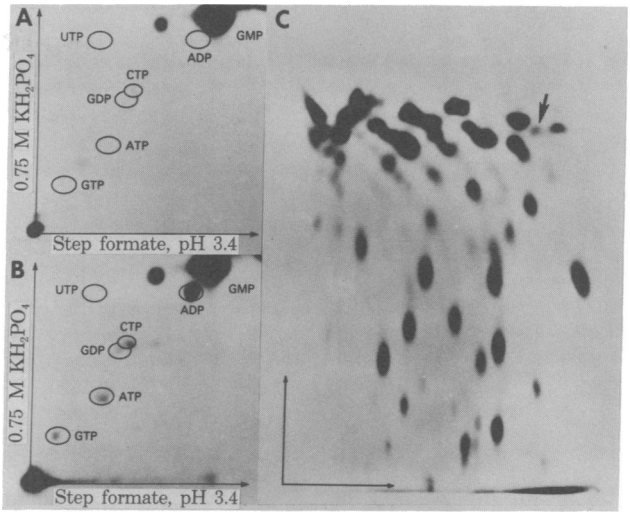

FIG. 3. Autoradiographs of two-dimensional separations of nuclease P1 digests (*A* and *B*) and of a ribonuclease T1 digest (*C*) derived from the *cro-y-cII* region. The RNAs were obtained after infections with $\lambda cI_{14}cII_{28}cIII_{611}$ (*A*) and λcI_{14} (*B* and *C*). For these experiments, RNA was isolated by the modified hybridization procedure described in *Materials and Methods* to minimize losses of the terminal polyphosphate moieties of 5′-nucleotides. The direction of development using the two different solvents is shown by arrows. The positions of various marker nucleotides are indicated by circles. The radioactivity appearing at the edge of the circle designated CTP (*B*) does not coincide exactly with the position of the marker nucleotide CTP. Moreover, the intensity of this spot was variable in different experiments. The oligonucleotide marked with an arrow in *C* has been identified by secondary analysis as ppApGp.

Characterization of the 5′ end of the leftward transcript

To determine whether the *cII*-dependent leftward RNA represents a primary transcription product from promoter p_E, it was necessary to characterize the 5′-terminal nucleotide residue of this RNA and to locate its position precisely within the DNA sequence. If the 5′ end of this RNA represents an *in vivo* transcription start site, then this terminal nucleotide should contain a 5′ polyphosphate moiety [e.g., (p)ppN. . .].

Initial experiments (data not shown) indicated that the hybridization conditions used to obtain the RNAs shown in Fig. 2 resulted in substantial losses of the 5′-terminal polyphosphate moiety from nucleoside 5′-di- and triphosphates. To minimize these losses, we reduced the time of exposure of the RNA to high temperature, as described in *Materials and Methods*.

By using these new hybridization conditions, RNA complementary to the *cro-y-cII* region of λ was isolated in parallel from cells infected with either λ *cII*⁺ or λ *cII*⁻ phage. The ^{32}P-labeled RNA obtained was then digested to mononucleotides with nuclease P1 and the products were resolved by thin-layer chromatography. The results, shown in Fig. 3, indicate that very little di- or triphosphorylated nucleotide [i.e., (p)ppN] is associated with the RNA obtained from the λ *cII*⁻ infection (*A*). However, in marked contrast, the RNA isolated from the λ *cII*⁺ infection yielded a significant amount of the polyphosphorylated nucleotides pppA and ppA (*B*). In addition, a small but significant amount of pppG and ppG is also observed. More than 95% of the radioactivity that was associated with nucleoside di- or triphosphates was *cII* dependent. Thus, the appearance of both the major (p)ppA nucleotide and the minor (p)ppG nucleotide coincides with the appearance of the *cII*-dependent leftward RNA. We conclude that this RNA represents a primary transcription product that initiates predominantly with (p)ppA and less frequently with (p)ppG.

To position this transcription start site precisely in the nucleotide sequence between residue positions 320 and 330 (see Fig. 4), RNA obtained in the same way as that used for nuclease P1 analysis was digested with T1 ribonuclease and fingerprinted in two dimensions. A major T1 oligonucleotide with the sequence ppApGp was found only in the RNA fingerprint made from the λ *cII*⁺ infection (Fig. 3*C*). The sequence of this oligonucleotide is consistent with the 5′-nucleotide identification and unambiguously positions the 5′ end of the major transcript at position 321 of the sequence shown in Fig. 4. The pppG residue could be assigned to a minor transcription start site at the adjacent residue position 320.

Effects on p_R transcription

We have compared the levels of p_R-promoted rightward transcription in the presence (λ *cII*⁺ infection) and absence (λ *cII*⁻ infection) of leftward transcription. Oligonucleotides characteristic of the p_R and p_E transcripts were eluted from the thin-layer chromatography plates and the amount of ^{32}P associated with each of them was determined. To permit comparison between parallel experiments, these values were then normalized to the radioactivity incorporated into total RNA in each experiment. We assumed that hybridization and recovery efficiencies were the same. The results (Table 1) indicate that the transcript initiated at p_R is present in approximately half the amount during the λ *cII*⁺ infection compared to the amount during the λ *cII*⁻ infection. It thus appears that the activation of the leftward transcription results in a corresponding decrease in rightward transcription from promoter p_R.

* Nucleotide positions are numbered sequentially starting with the first nucleotide of the p_R transcript (30, 31).

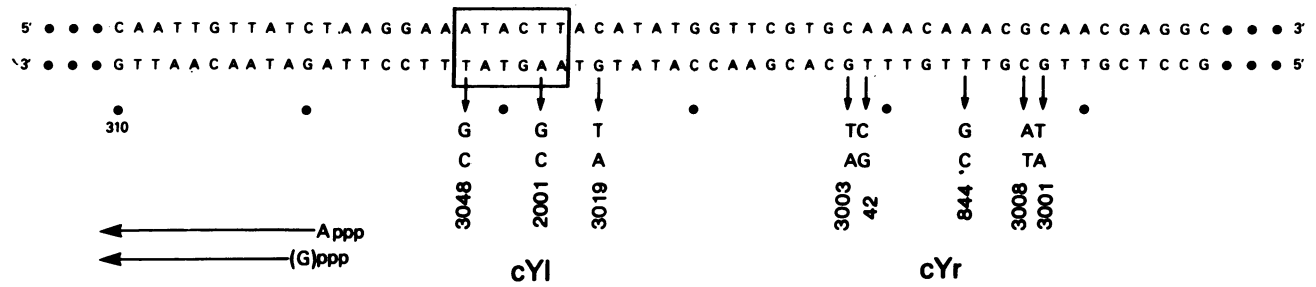

FIG. 4. p_E promoter as defined by transcriptional startsites and *c*Y mutations. Residue numbers are the same as those used in ref. 30. Arrows indicate the direction of transcription. pppA and pppG represent the major and minor 5'-terminal nucleotides of the p_E transcript, respectively. *c*Yl and *c*Yr define the regions affected by the left and right clusters of *c*Y mutations, respectively. These mutations have been characterized previously (17). The putative "−10" hexamer sequence of the p_E promoter is indicated by a box.

DISCUSSION

Initiation Site of *c*I Transcription. We have isolated and identified a specific RNA transcript that begins at a site within the *y* region and extends leftward toward the phage repressor (*c*I) gene. Synthesis of this RNA is completely dependent upon the products of phage genes *c*II and *c*III, and it requires a wild-type *c*Y regulatory site. Identification of the 5'-terminal nucleotide of this transcript indicates that the RNA predominantly starts with (p)ppA and less frequently with (p)ppG (Fig. 4). Further characterization of the 5' terminus of the transcript positions the pppA residue unambiguously at nucleotide residue 321 and the pppG at position 320 within the sequence of the *y* region. We conclude that this site defines the transcription startpoint(s) for the promoter responsible for the establishment of repressor synthesis, p_E.

No transcription from the 200-bp region immediately upstream of this site was detected. The sensitivity of the autoradiography would have allowed detection of 1% of the level found downstream, and the effects of ribonuclease digestion or processing of RNA were minimized by labeling for a short time, 1.5 min. Thus we conclude that little, if any, transcription occurs through the *cro-y-c*II region from promoters upstream of p_E (e.g., p_O), during the time of establishment of repression. These results contradict those found by Honigman *et al.* (20) and are incompatible with the model that proteins cII/cIII act as antitermination factors in the *y* region.

p_E: A Positively Regulated Promoter. RNA polymerase is known to interact with two major regions of a promoter. These regions have been defined by RNA polymerase binding studies, by mutations, and by comparison of sequence homologies among various promoters. The "−35" region, which is defined as the region located about 35 bp before the initiation site, contains a core sequence generally homologous to the sequence TTGACA (34, 35). This region is thought to be important for initial recognition by RNA polymerase. The other region, called "−10" region, is centered about 10 bp preceding the initiation site and also contains a highly conserved core sequence, typically TATAAT (36, 37). This region probably serves to bind and align RNA polymerase on the DNA for transcription initiation. The sequence ATTTGT, immediately preceding the hexamer TATAAT, is also found in various promoters and has been shown to be important for promoter function. Comparison of the p_E promoter with other promoters indicates little homology to the usually conserved bases in the "−35" and "−10" regions (for review, see ref. 38).

Table 1. Comparison of leftward and rightward transcription through the *cro-y-c*II region

λ	Mole ratios of transcripts p_R	p_E
*c*II⁻	2	<0.06
*c*II⁺	1	6

The amount of ^{32}P associated with oligonucleotides characteristic of the p_R- and p_E-promoted transcripts (see Fig. 2) was determined as cpm per phosphate and normalized to the amount incorporated into total RNA in each experiment. The numbers in this table represent ratios determined from the average of eight oligonucleotides from each of the two transcripts. The average cpm per phosphate in the p_E transcript was 360. Total incorporation into RNA was 4.4×10^7 cpm for the λ *c*II⁺ infection and 3.3×10^7 cpm for the λ *c*II⁻ infection. Between 6% and 8% of total RNA hybridized to λ DNA.

Some promoters do not bind RNA polymerase unless an additional regulatory protein is present. The *E. coli* cyclic AMP receptor protein (CRP) (39–41) and the cI repressor (8) are such proteins; both bind to DNA upstream of promoter initiation sites and enhance the polymerase–promoter interaction. Two results suggest that the p_E promoter is also positively regulated. (*i*) *In vivo*, cII function is always required to activate *c*I expression from the p_E promoter. (*ii*) *In vitro*, purified RNA polymerase neither binds to nor initiates transcription from the p_E promoter region (42, 43; unpublished results). We propose, therefore, that cII protein is the positive regulator of p_E. We believe that cII protein must compensate in some way for the lack of homology of p_E with conventional RNA polymerase binding regions.

Additional observations suggest that the *c*II product interacts with the "−35" region of the p_E promoter. There are two classes of p_E promoter mutations, which map in two distinct clusters, namely *c*Yl, between positions −7 and −14, and *c*Yr, between positions −27 and −37 relative to the initiation site (Fig. 4). Wulff *et al.* (17) have shown that lysogenization by *c*Yl mutants is still stimulated by cII protein, whereas this activation is not observed with *c*Yr mutants. The positions of the mutated bases in *c*Yr do not reveal a relationship between this sequence of the p_E promoter and the conventional RNA polymerase recognition site (17, 38). These mutations most likely define the site essential for positive regulation.

By analogy, the regulatory region for *c*II-dependent expression of the *int* gene might be expected to contain sequence information similar to p_E. The sequence of the putative *int* promoter, p_I, has been determined, and it does contain a sequence homologous in 11 of 14 bp to the p_E promoter (44, 45). This region of homology is located in similar positions preceding the p_E and p_I initiation sites. In the p_E promoter, these 14 bp encompass all the *c*Yr mutations. Although there is as yet no direct evidence that cII binds to the DNA of the *c*Yr site, both genetic and sequence analyses support this possibility.

How do *c*Yl mutations affect the p_E promoter? Their positions relative to the initiation site suggest that they might interfere with RNA polymerase binding in the "−10" region. The most important bases in the conventional "−10" hexamer

TATAAT are A in position 2 and T in position 6 (38). The sequence AAGTAT, extending from position −12 to −7 relative to the p_E initiation site, contains these two bases in the correct location. These same two bases are altered by *c*Yl mutations 2001 and 3048, respectively. A third *c*Yl mutation, 3019, alters the G in the ATATGT sequence preceding the hexamer AAGTAT. These three *c*Yl mutations change conserved sequences likely to be involved in RNA polymerase interaction.

Convergent Transcription Between Promoters p_E and p_R. Our results indicate that the activation of leftward transcription from the p_E promoter is coincident with a reduction to one-half in rightward transcription from promoter p_R during the period from 8 to 9.5 min after infection. This finding is consistent with previous reports (33, 46) that the cII product activates *c*I gene expression and concomitantly reduces expression of the late lytic genes, which are under the control of rightward transcription. We propose that this form of regulation results directly from the effects of convergent transcription.

Ward and Murray (47) have shown recently that, when convergent transcription occurs, the stronger promoter appears to gain an advantage over a weaker promoter and progressively reduces transcription from the latter. Our results are consistent with this idea—i.e., the stronger p_E-promoted transcript (Table 1) turns down the converging weaker transcription from promoter p_R.

Thus, convergent transcription from these two promoters is an important mechanism by which phage λ can control its development. High levels of p_E transcription favor the lysogenic response, whereas low levels of p_E transcription favor the lytic response. Infection under most physiological conditions leads to intermediate levels of p_E transcription, which allows appreciable numbers of infected cells to enter either developmental pathway.

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