Autoregulation and Function of a Repressor in Bacteriophage Lambda

Interactions of a regulatory protein with sequences in DNA mediate intricate patterns of gene regulation.

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Genes can be regulated by the interaction of proteins with specific sequences in DNA (*1*). Proteins called repressors specifically turn off transcription, and positive regulatory proteins enhance specific transcription. In this article we describe the complete sequence of two control regions in the DNA of a bacteriophage. We show how interaction of these sequences with a regulatory protein mediates intricate patterns of gene regulation. In particular, we show that one of these sequences is arranged so that a single protein can function both as a positive and a negative regulator. Moreover, we argue that this same control region may contain information important for posttranscriptional control.

Coliphage lambda (λ) codes for a repressor that turns off transcription of the other bacteriophage genes, including those whose expression causes lytic phage growth (*2–4*). In this way repressor maintains the genome of a potentially lethal phage (prophage) integrated inertly in the chromosome of its bacterial host. Such a lysogenic bacterium, as it is called, will lyse and produce phage if the repressor is inactivated. Various agents, including ultraviolet light, cause repressor inactivation by a mechanism that is not well understood (*5*) and which we do not consider further in this article.

Repressor controls its own synthesis, both positively and negatively [autoregulation or autogenous control (*6*)] (*7, 8*). This action of repressor, in addition to those alluded to above, is effected by interaction of repressor with control regions in λ DNA. There are two such control regions and each includes sites recognized by repressor (the operators) and by RNA polymerase (the promoters). We consider explicitly three examples of gene regulation involving the repressor, or its gene (*c*I) (see Fig. 1).

1) The repressor binds to two operators, called o_L and o_R, and blocks initiation of transcription at the corresponding promoters p_L and p_R. Repressor bound to o_L blocks leftward transcription of gene *N*, and repressor bound to o_R blocks rightward transcription of the gene called *tof* or *cro*. Action of repressor at the two operators suffices to turn off most (about 50) of the phage genes. This is in part because the product of gene *N* is required for expression of most of the other phage genes (*2*). Recent experiments have revealed how repressor bound at each operator effects control of the neighboring genes and we shall review that argument.

2) The *c*I gene is transcribed in two modes. In the lysogenic state, transcription begins near the right end of *c*I (near o_R) at the promoter called p_{RM} [promoter for repressor maintenance (*9, 10*)]. This transcription (*8*) is itself subject to both positive and negative control by repressor. Thus, the amount of repressor in lysogens is carefully regulated. We describe our current understanding of the molecular mechanisms of this autogenous control.

3) Upon infection of a nonlysogenic cell, *c*I transcription begins about a thousand bases to the right of *c*I and hence well to the right of o_R, at a promoter called p_{RE} (promoter for repressor establishment) (*10–12*). [Transcription beginning at p_{RE} requires the positive regulatory factors coded by the phage *c*II and *c*III genes (*13*). Once repression has been established, transcription of *c*II and *c*III is turned off by repressor, and *c*I is no longer transcribed from p_{RE}. The mechanism of action of *c*II and *c*III is not understood and is not considered further here.] p_{RE} directs the synthesis of five- to tenfold more repressor, per genome, than does p_{RM} (*10*), and provides the large burst of repressor necessary for the establishment of lysogeny. We present evidence that suggests a novel mechanism of posttranscriptional regulation that explains how p_{RE} directs the synthesis of more repressor than does p_{RM}.

Before considering these three issues we describe our understanding of the structures of the λ operators, promoters, and repressor.

Operator Structure

The most striking aspect of the λ operators is that each contains three repressor binding sites (o_L1, 2, 3; o_R1, 2, 3). The sequences specifically recognized are 17 base pairs long and are separated by "spacers" rich in A (adenine) and T (thymine) three to seven base pairs long. The terminal binding sites o_L1 and o_R1, which are adjacent to the controlled genes *N* and *cro*, bind repressor with a higher affinity than do the remaining sites. The complete nucleotide sequences of the λ operators are shown in Fig. 2. In Fig. 3 is shown a cartoon of these sequences that emphasizes several important features. The evidence for the preceding statements may be summarized as follows:

1) At each operator, repressor protects from pancreatic deoxyribonuclease digestion fragments roughly 25, 50, and 80 base pairs in length (*14*). The size of the protected fragment increases in steps as the ratio of repressor to operator in the digestion mix is increased (*14, 15*).

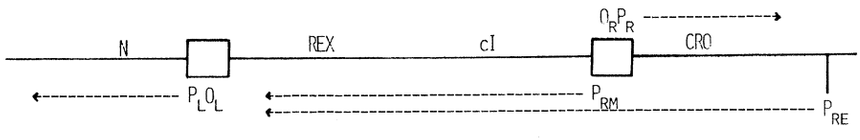

Fig. 1. Schematic representation of transcriptional patterns in a portion of the λ genome. The arrows show the directions of transcription of genes *N*, *cro*, *c*I, and *rex*. Genes *c*I and *rex* are transcribed either from the promoter p_{RM} in lysogens or from p_{RE} after phage infection of nonlysogens. o_Lp_L represents the "leftward" and o_Rp_R the "rightward" operator promoter.

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The smallest fragment from each operator corresponds to o_L1 or o_R1, plus a few adjacent nucleotides, as shown by analysis of pyrimidine tracts (*14, 15*). [Somewhat more complex results of repressor protection experiments have been described (*15, 16*). In contrast are our current findings, which we believe are more reliable because we used a more highly purified repressor. Presumably the previous results were complicated by the presence of low levels of uncharacterized contaminants in the repressor.]

2) Various restriction endonucleases cleave within each operator. For example, the enzyme *Hin*dII cuts once in each operator. Four of the fragments produced by *Hin*dII cleavage of λ DNA bear different portions of o_L and o_R, and each of these binds repressor (*17*). These experiments show directly that each operator contains more than one site that can independently bind repressor.

3) Virulent mutants of phage λ grow in λ lysogens. These mutants have lost their sensitivity to repressor in vivo. Mutations that decrease the repressibility of phage have been located within the sequences o_L1, o_L2, and o_R2. Each of these mutations decreases the affinity of a binding site for repressor in vitro (*18–20*). Mutations in the spacers have no effect on repressor affinity, but they do affect the action of RNA polymerase at the corresponding promoter (*8, 21*).

4) There is a striking similarity between the sequences of the binding sites. In Fig. 4, the six sites are aligned and the frequency with which a given base appears at each position is tabulated. The bases changed by various operator mutations are boxed. Each site contains elements of twofold rotational symmetry (*3, 19*), but it is highly unlikely that this symmetry results in the formation of hairpin loops that are recognized by repressor (*15*). Presumably these operator symmetries correspond to symmetric features of the repressor oligomer. Each repressor binding site consists on one side of the sequence 5′-TATCACCGC-3′ (C, cytosine; G, guanine), or a sequence differing by two bases, plus, on the other side, a related but more variable sequence. This may imply that the detailed interaction of repressor with operator is not completely symmetric. Operator mutations change bases that are largely conserved amongst the various sites.

5) The extent of methylation of G and A residues in DNA by dimethyl sulfate may be used to identify those bases protected by a bound protein (*22*). The ring nitrogen (N-7) methylated on G lies in the major groove of the helix,

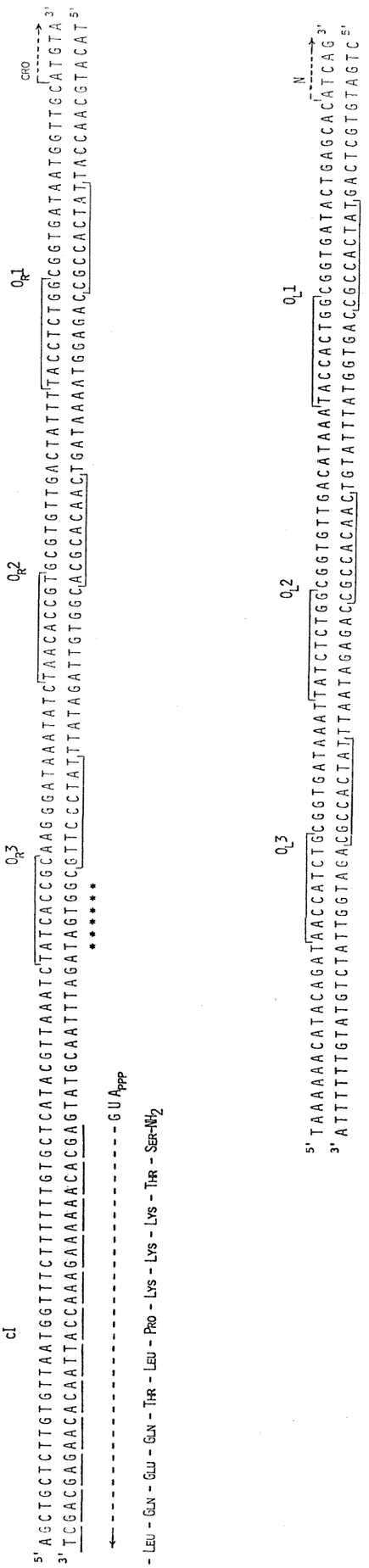

Fig. 2. DNA, RNA, and protein sequences in and around the two control regions of phage λ. The repressor binding sites o_L1, 2, and 3 in o_L (left operator) and o_R1, 2, and 3 in o_R (right operator) are set off in brackets. The start points of transcription of genes *N*, *cro*, and *c*I are indicated (*8, 49*). Also shown are amino terminal residues of the repressor. Six bases on o_R 3, presumed to code for a strong ribosome binding site for *c*I, are marked with an asterisk. The o_L has been reversed from its orientation in Fig. 1. Most of the DNA sequences, plus that of *c*I mRNA, were reported previously (*18, 50*). The new sequences, which include that of o_L3, were determined in our laboratory (*14*). The sequence to the left of o_R3 corrects that tentatively suggested (*21*, figure 1). Walz *et al.* (*51*) have independently determined the sequence of *c*I mRNA and their sequence agrees with ours. See Fig. 6 for the amino acid sequence of repressor.

whereas the N-3 methylated position on A lies in the minor groove. Lambda repressor protects G's but not A's from methylation, and, as expected, only those G's located within the designated repressor binding sites (*14*). This result also suggests that lambda repressor specifically contacts DNA in the major groove (*23*).

Promoter Structure

A promoter is defined as a DNA sequence necessary for recognition and binding of RNA polymerase and for initiation of transcription. The promoters p_L, p_R, and p_{RM} denote, respectively, promoters for genes *N*, *cro*, and *c*I transcription. Figure 3 shows the regions of DNA protected from deoxyribonuclease digestion by RNA polymerase bound at these promoters. As shown first with p_R, the protected fragment is about 45 base pairs long when pancreatic deoxyribonuclease is used, and transcription begins roughly in the middle of this protected sequence (*24*). A similar relation between polymerase-protected fragments and transcription start points has been found in other cases where pancreatic deoxyribonuclease has been used (*25*). We have recently repeated these polymerase protection experiments at p_R, using λ exonuclease and the single-strand specific nuclease S1 in place of pancreatic deoxyribonuclease (*14*). Under these conditions the protected piece is roughly 65 base pairs long, and its approximate extent is indicated on the figure. We comment below on the fact that polymerase-protected fragments overlap repressor binding sites in the operators.

Two promoter mutations have been sequenced (*8, 21*). One, located in the spacer between o_L1 and o_L2, damages p_L.

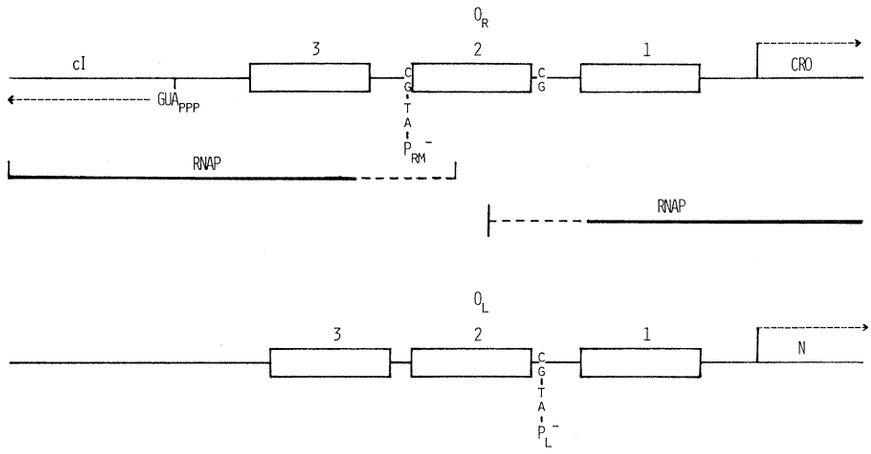

Fig. 3. Cartoon of the lambda operators and a portion of genes *N*, *cro*, *c*I, and *rex*. The boxes show the positions of the 17-base pair repressor binding sites. The start points of *cro*, *N*, and p_{RM}-directed *c*I transcription are indicated. The approximate positions of the RNA polymerase binding sites at p_L, p_R, and p_{RM}, defined as the DNA protected from deoxyribonuclease digestion by polymerase, are shown. The extents of the fragments protected (solid or dashed lines) depend on the particular deoxyribonuclease used in the protection experiment (see text). Two promoter mutations are shown.

The other, located in the spacer between o_R2 and o_R3, damages p_{RM}. The former is 31 and the latter 33 base pairs from the respective start points of transcription, and each changes the sole G · C in a spacer to A · T. We also know that a promoter mutation occurs in p_R within a few base pairs of the position analogous to that of the p_L mutation, but the exact base change has not been determined (*26*). As indicated in Fig. 3, the RNA polymerase protected fragment generated by pancreatic deoxyribonuclease digestion does not include the regions in which these promoter mutations occur. It is not surprising, therefore, that these fragments do not bind polymerase. In contrast, the larger protected pieces obtained by λ exonuclease and nuclease S1 treatment include these regions, and these fragments bind polymerase and direct transcription (*14*). We do not know why digestion of polymerase-DNA complexes with different nucleases yields fragments of different sizes.

Lambda Repressor

The lambda repressor is an acidic protein, whose monomer has a molecular weight of about 26,000 (*27*). These monomers are in concentration-dependent equilibrium with dimers and tetramers. The repressor binds tightly to DNA as an oligomer, but it is not known whether dimers or tetramers bind to the sites within the operators (*16, 28*).

Repressor is produced in small amounts in ordinary lysogens, about 200 monomers per cell (*10*). We have constructed in vitro a recombinant DNA molecule that contains the *c*I gene read from two *lac* promoters (*29*) (Fig. 5). This recombinant is incorporated in a plasmid, and bacteria carrying this plasmid (pKB252) overproduce repressor some 50- to 100-fold. Sufficient quantities of repressor have been isolated to permit sequence analysis. The first 51 amino acids of the sequence are shown in Fig. 6. A striking feature of the amino terminus of repressor is the strong clustering of basic residues. Although arginine and lysine constitute about 10 percent of the total residues, they account for 33 percent of the 27 amino terminal residues. It has been suggested that amino terminal residues of repressor make specific contacts with operator DNA (*30*), as has also been suggested for the *lac* repressor (*31*).

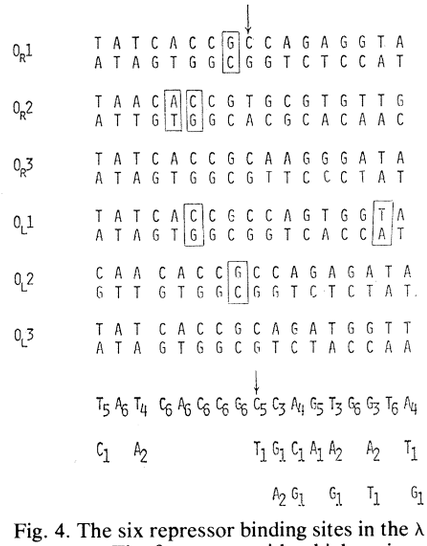

Fig. 4. The six repressor binding sites in the λ operators. The frequency with which a given base appears in each position is tabulated. Thus, for example, in position 1, *T* appears five times, *C* once. The sites have been oriented to reveal their similarities. The arrows indicate the axis of partial twofold symmetry in each site. Base pairs changed by mutations that decrease repressor affinity are boxed.

We now consider the three examples of gene regulation mentioned earlier.

Repressor Control of *N* and *cro*

The two terminal repressor binding sites in o_R (o_R1 and o_R2) and in o_L (o_L1 and o_L2) mediate repression of *cro* and *N*, respectively. This was deduced from the fact that mutations that render *cro* transcription constitutive—that is, mutations that reduce repression at that operator—have been found in o_R1 and o_R2; mutations with a similar effect on *N* transcription have been found in o_L1 and o_L2. At each operator, mutation of two sites has a more dramatic effect on repression than does mutation of either site alone (*8, 18*). RNA polymerase and repressor binding sites overlap in each operator (see Fig. 3), and repressor excludes binding of polymerase. Apparently repressor bound to two sites excludes polymerase more efficiently than does repressor bound to a single site. Repressor blocks transcription only if added to the template before RNA polymerase (*8, 32*).

Self Regulation of *c*I

Negative control. Repressor bound to o_R3 turns off transcription of *c*I. This was deduced as follows. RNA was transcribed in vitro from a DNA fragment bearing p_{RM} and a portion of the *c*I gene, and the *c*I transcript was identified by several criteria (*8*). Most important is that this message was not produced if the template bore a mutation that prevents transcription from p_{RM} in vivo. The sequence of the *c*I transcript corresponds to the DNA sequence as shown in Figs. 2 and 3. Relatively high concentrations of repressor turn off transcription of this message in vitro (*8*) and in vivo (see below). Mutations in o_R1 and o_R2 do not drastically affect this repression as measured in vitro (*33*). As at p_R, repressor blocks transcription only if added to the template before RNA polymerase (*8*).

Positive control. Repressor bound to o_R1 enhances transcription of *c*I (*8, 14*). The efficiency with which *c*I is transcribed in vitro can be increased five- to tenfold by the addition of repressor (*34*). This effect requires an intact o_R1. The mechanism of this positive effect is unknown—in particular, we do not know the role of o_R2—but two possibilities are as follows. (i) RNA polymerase bound to

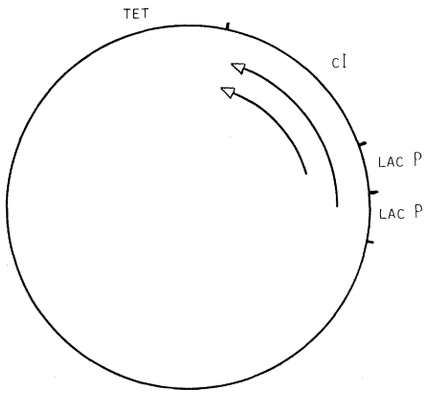

Fig. 5. Structure of the repressor-overproducing plasmid pKB252. The plasmid carries the λ *c*I gene adjacent to two copies of the promoter from the *lac* operon. *c*I is transcribed from these tandem *lac* promoters. The orientation of the *lac* promoters, *c*I, and the gene that confers resistance to tetracycline (*tet*) is shown. The λ and *lac* sequences were inserted into the plasmid pMB 9 in vitro (*29*).

p_R prevents, by steric inhibition, other polymerase molecules from binding to p_{RM}; repressor bound to o_R1 prevents polymerase binding to p_R, but does not block access of polymerase to p_{RM} (*24*). (ii) Repressor bound to o_R1 directly enhances polymerase binding at p_{RM}, either by providing a protein-protein contact, or by subtly altering DNA structure. We note that the distance from the center of o_R1 to the start point of transcription of *c*I is about the same as that between the center of the CAP (catabolic gene activator protein) binding site and the start point of transcription of the *lac* operon (*35*). The CAP enhances transcription of the *lac* gene, but the mechanism is not understood.

We favor some version of the second model, but we feel the evidence is not conclusive (*36*).

Translational Control of *c*I

Why does transcription of *c*I initiated at p_{RE} produce more repressor than does transcription from p_{RM}? We had anticipated a simple answer: p_{RE} is a more efficient promoter than p_{RM}, and hence more *c*I transcripts are read from p_{RE}. This statement may be true, but recent evidence indicates that a more important factor is differential translation of the messages initiated at the two promoters. Figures 2 and 3 show that the codon corresponding to the amino terminus of repressor is found immediately adjacent to the 5′ terminal AUG (U, uracil) of the *c*I message. This is remarkable in that all messages analyzed heretofore contain leaders of variable length, preceding the AUG or GUG translational start signals (*37*). These leaders have been found to contain short sequences that are complementary to sequences at the 3′ end of 16*S* ribosomal RNA. It has been argued that pairing of these complementary sequences promotes binding of messages to ribosomes, and hence efficient translation (*37*). Our finding that the *c*I message transcribed from p_{RM} bears no leader suggests that it may be translated at low efficiency. In contrast, we note that beginning 12 bases to the right of the translational start point there is a six-base sequence complementary to a sequence at the 3′ end of 16*S* ribosomal RNA (see bases marked with an asterisk in Fig. 2). This sequence should be present in the *c*I message transcribed from p_{RE} and should function as a strong ribosome binding site. Smith *et al.* (*38*) have found that the *c*I message transcribed from p_{RE} is processed in vivo, but that the cleavage site is to the right of the proposed ribosome binding site. We conclude, therefore, that message transcribed from p_{RE} bears a strong ribosome binding site and is translated more efficiently than is message transcribed from p_{RM} (*39*).

Further Analysis of p_{RM} and Its Transcript

We have constructed a hybrid operon that dramatically illustrates the positive and negative effect of repressor on transcription initiated at p_{RM}. Furthermore, properties of the hybrid reveal that the p_{RM} transcript is initiated relatively efficiently and, as was predicted, is translated relatively inefficiently. This hybrid operon, in which *c*I, *trp*A, and *lac*Z are all transcribed from p_{RM}, was constructed by recombination in vitro and is carried on a transducing phage (*40*). The *lac* and *trp* promoters are missing in this phage, but the respective ribosome binding sites are present. On the basis of experiments reported by Reznikoff *et al.* (*41*), we expect that *lac* mRNA transcribed from p_{RM} in our particular transducing phage would be translated as efficiently as is ordinary *lac* mRNA transcribed from the *lac* promoter. We can therefore conveniently measure p_{RM} function in lysogens of this phage by assaying β-galactosidase, the product of the *lac*Z gene, and we can compare this function directly with that of the wild-type *lac* promoter. In order to measure the function of p_{RM} in the absence of repressor, we have crossed into this phage a λ*c*I mutation. As is indicated in Fig. 7, the transducing phage carries repressor genes from two different phages (λ and 21) and because the λ repressor does not control lytic function in this phage, λ*c*I mutants lysogenize normally. Finally, we are able to study the effect of high concentrations of repressor on p_{RM} function by adding to lysogens of the trans-

Table 1. p_{RM} directed synthesis of β-galactosidase. Lysogens of the phage shown in Fig. 7 (*c*I$^+$) and of the *c*I$^-$ derivative described in the text were grown in glycerol minimal medium and assayed (*52*) for β-galactosidase. Also assayed were a lysogen bearing the *c*I$^+$ phage and the plasmid pKB252 which overproduces λ repressor, and, for comparison, a lysogen bearing a *lac*Z gene which is maximally expressed from the *lac* promoter (p*lac*5). Results are given in the units of Miller (*52*).

Strain	β-Galactosidase activity
*c*I$^-$	100
*c*I$^+$	2,650
*c*I$^+$ and pKB252	210
p*lac*5	13,650

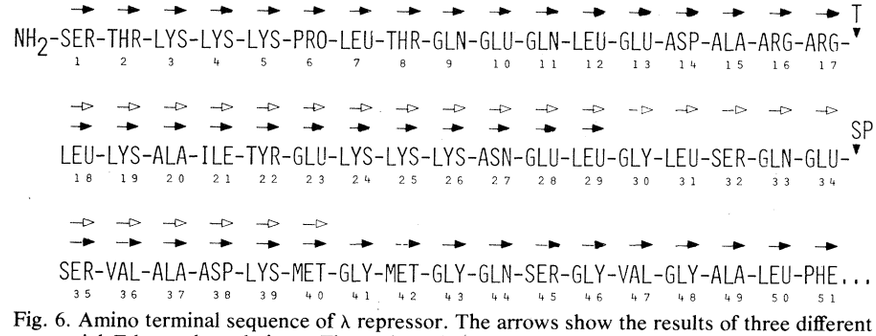

Fig. 6. Amino terminal sequence of λ repressor. The arrows show the results of three different sequential Edman degradations. These degradations were performed on intact repressor, a peptide generated by tryptic cleavage at Arg^{17}, and a peptide generated by staphylococcal protease cleavage at Glu^{34} (*14*). Beyreuther and Gronenborn (*30*) have presented a similar sequence, but with Asn^{14}, Ser^{40}, and Thr^{42} instead of the residues reported here. Abbreviations for the amino acid residues are Ala, alanine; Arg, arginine; Asp, aspartic acid; Asn, asparagine; Glu, glutamic acid; Gln, glutamine; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Pro, proline; Phe, phenylalanine; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

ducing phage the repressor-overproducing plasmid pKB252.

The β-galactosidase levels in various lysogens of our transducing phage reflect the autogenous regulation at p_{RM} (Table 1). The results are qualitatively as follows. In the absence of repressor, only a low level of β-galactosidase is made; the presence of one gene dosage of repressor greatly increases enzyme production, and excess repressor turns down enzyme synthesis. These results support the conclusion drawn previously that small amounts of repressor enhance, and larger amounts decrease, transcription initiated at p_{RM}. Moreover, we see that in the presence of ordinary λ-lysogen levels of repressor, p_{RM} directs the synthesis of about 20 percent as much β-galactosidase as does a fully induced *lac* promoter. These facts also give us an approximate measure of the relative efficiency of translation of *c*I message transcribed from p_{RM}. An ordinary *Escherichia coli* bacterium fully induced for *lac* contains at least 20,000 monomers of β-galactosidase (*42*). This fact, taken with the results shown in Table 1, indicate that p_{RM} directs the synthesis of about 4000 monomers of β-galactosidase. An ordinary λ lysogen contains about 200 monomers of repressor, from which we conclude that the *c*I message is translated no more than 5 percent as efficiently as is *lac* mRNA. We imagine that this low level of translation is caused by the lack of a strong ribosome binding site on the p_{RM} directed *c*I messenger.

Recapitulation and Additional Considerations

Let us consider the action of repressor at one operator, o_R. This operator contains three repressor binding sites designated o_R1, o_R2, and o_R3. Because o_R1 has the highest repressor affinity, at low concentrations repressor will be bound preferentially to o_R1. This has the dual effect of decreasing rightward transcription of gene *cro* and of enhancing leftward transcription of the repressor gene, *c*I. At higher repressor concentrations o_R2 is filled, further repressing transcription of *cro*; and at very high repressor concentrations o_R3 is filled and transcription of *c*I ceases. This sequential interaction of repressor with sites within a single controlling sequence mediates negative control of a function required for lytic growth of the phage (*cro*) and autoregulates, both positively and negatively, production of repressor. Part of this same operator (o_R3) codes for a sequence that

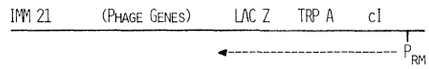

Fig. 7. Prophage map of transducing phage bearing a *lac* gene transcription of which is directed by p_{RM}. The phage carried intact *lac*Z, *trp*A, and λ*c*I genes. In a lysogen, all three are transcribed only from p_{RM} (*40*).

apparently ensures efficient translation of *c*I, but that sequence is contained in the *c*I message only if transcription begins at one of two of the possible *c*I promoters (p_{RE}). The left operator (o_L) is similar in structure to o_R; repressor bound to o_L1 and o_L2 turns off transcription of another gene (*N*) required for lytic growth; the function of o_L3 is not known.

The functions we have ascribed to o_L and o_R are not exhaustive. We have strong reason to believe that another repressor, the product of the *cro* gene, binds to o_L and o_R during lytic growth to turn down synthesis of *N*, *cro*, and *c*I, but how it does so remains to be seen (*43*). Moreover, the *N* protein is known to recognize some sequence in or near these operators, and to render RNA polymerase immune to the blockade found at the end of certain genes (*44*). We have not yet elucidated all the functions mediated by these remarkable regulatory sequences.

Addendum

The molecular mechanisms of gene regulation we have discussed raise certain biological questions to which we wish to suggest possible answers.

1) Why is repressor synthesis subject to negative and positive autoregulation in lysogens? We begin with the premise that it is important to maintain repressor concentrations over a rather narrow range. Too much repressor would make it difficult for the phage to induce under emergency conditions, and too little repressor would put the lysogen at risk of inducing unnecessarily. The combination of positive and negative autoregulation ensures that, for example, a shift up or down in the growth rate will not seriously alter repressor levels. Synthesis of many other proteins is subject to a form of positive and negative regulation that senses the growth conditions of cells. For example, many genes that code for catabolic enzymes (such as the *lac*Z gene) are transcribed only if cyclic AMP is present in sufficiently high levels to activate the positive regulatory protein CAP (*45*). A shift down in growth rate (catabolite repression) decreases *lac*Z transcription by depleting cyclic AMP, whereas a shift up in growth rate has the opposite effect. The *c*I gene does not utilize a cyclic AMP–sensitive device for regulating transcription at p_{RM}. Rather, homeostasis is achieved by combining positive and negative self regulation.

2) We have argued above that *c*I mRNA transcribed from p_{RM} is translated inefficiently because it lacks a leader containing a strong ribosome binding site. Why is this heretofore undescribed form of translational control used to limit the total repressor that can be synthesized in a lysogen? Why not, for example, design p_{RM} so that the *c*I message is transcribed at low maximal efficiency? One possible answer might be found in the fact that the *c*I operon is polycistronic. That is, the transcript of a second gene, called *rex*, is an extension of the *c*I message (*9*) (see Fig. 1). In a λ lysogen the *rex* product prevents growth of *r*II mutants of T4, but that is probably not its primary function. It is possible that *rex* is required in higher levels in lysogens than is repressor. Possibly the *rex* portion of the message contains its own ribosome binding site and is translated at high efficiency. (This is plausible in view of the fact that the *lac*Z message, bearing its own ribosome binding site, is translated at high efficiency when fused to *c*I.) Thus, specific protein levels can be maintained by differential translation of different portions of the same mRNA molecule.

The fact that the λ repressor gene is transcribed efficiently but translated inefficiently has an interesting consequence, namely, that λ repressor will be produced at a constant rate throughout the life cycle of a lysogenic bacterium (*46*). Apparently this is not the case for the *lac* repressor. The *lac* repressor (*i*) gene messenger bears a leader with a ribosome binding site (*47*), and we expect that each such messenger, like most other messengers, would be translated repeatedly. Thus bursts of repressor would be produced whenever the *i* gene is transcribed. However, only a low level of *lac* repressor is found (about 20 to 40 monomers per cell), which suggests that the *i* gene is transcribed only once or twice per cell generation (*48*). Thus *lac* repressor levels must fluctuate during the cell cycle. It is possible that λ lysogens cannot tolerate similar fluctuations in the concentration of λ repressor. It remains to be seen whether the synthesis of other regulatory proteins is similar to that of λ*c*I.

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34. The stimulatory effect of repressor on transcription of *c*I can be mimicked by adding a large excess of RNA polymerase. The value given here for the stimulatory effect of repressor is found with RNA polymerase in about fivefold molar excess over template. We have considered the trivial possibility that repressor stimulates *c*I transcription by preventing RNA polymerase binding to p_R, thereby raising the effective concentration of polymerase. We believe this explanation unlikely in view of the following result. There was no stimulatory effect of repressor on *c*I transcription when the template consisted of a mixture of two DNA molecules, one of which bore a p_{RM}^- mutation, and the other of which lacked o_R1. Thus, p_{RM} must be located *cis* to o_R1 for a stimulation of *c*I transcription by repressor to occur.
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36. Model 1 predicts that initiation of transcription at p_{RM} would occur efficiently if p_R were missing from the template. However, in preliminary experiments using such a template, the so-called *Hin*d375 of (*8*), and with polymerase in fivefold molar excess, *c*I was transcribed at a low level. As was expected from the fact that *Hin*d375 lacks o_R1, repressor did not enhance this transcription. At higher ratios of polymerase to DNA, *c*I was transcribed as efficiently as was a template bearing intact o_R1 and p_R, indicating that p_{RM} is not defective in *Hin*d375. These results argue against model 1; also see (*34*).
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39. We have considered the possibility that the p_{RM} and p_{RE} messages bear different translation start points and that two different forms of repressor might be synthesized. We believe this unlikely for the following reason. We imagine that the protein we and Beyreuther and Gronenborn (*30*) have sequenced is analogous to p_{RE} repressor because, in both cases, transcription of *c*I begins well to the right of p_{RM}. We have purified repressor from a strain producing *c*I product from p_{RM}, and have found it to run with a mobility identical to the sequenced repressor on 12 percent polyacrylamide gels containing sodium dodecyl sulfate. We assume we could detect a difference of 10 to 15 amino acids. Moreover, from the sequence of repressor we know that there are no in-phase AUG or GUG codons in the DNA within 108 nucleotides of the terminal AUG. Since proteins ordinarily start translation at either AUG or GUG, the hypothetical second form of repressor would have to be at least 36 amino acids shorter than the one we have sequenced.
40. This phage was constructed by joining three DNA fragments in vitro, with the use of polynucleotide ligase. One fragment was the left half of a phage bearing the *trp-lac* fusion W205 [W. Barnes, R. Siegel, W. Reznikoff, *Mol. Gen. Genet.* **129**, 201 (1974); D. Mitchell, W. Reznikoff, J. Beckwith, *J. Mol. Biol.* **93**, 331 (1975)]. This fragment was generated by cleavage with *Hin*dIII, which cuts in the *trp*B gene. The second fragment was the right half of a derivative of λimm^{21}, whose only *Eco* RI site is to the left of the phage attachment site (kindly provided by R. Pastrana and W. Brammar). The third, and shortest fragment, which contains λ's *c*I and p_{RM}, extends from the *Hin*dIII site in *rex* to a *Hae*III site in *cro*. This *Hae*III site was converted to an *Eco* RI site (*29*).
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