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Biochemistry

Mechanism of action of the *cro* protein of bacteriophage λ

(λ repressor/*cI*/operators/transcription/dimethyl sulfate)

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ABSTRACT **The mechanism of action of *cro* protein was probed by measuring its ability to protect DNA against methylation by dimethyl sulfate and its effect on transcription *in vitro*. The *cro* protein binds to the same three sites in the right operator (O_R) of bacteriophage λ DNA as does the λ repressor. Dimethyl sulfate protection experiments reveal major groove contacts for both proteins, and *cro* protein protects from methylation a subset of those purines protected by λ repressor. These experiments also show that the relative affinity of these two proteins for the three operator sites is different: whereas λ repressor binds with an affinity $O_R1 > O_R2 > O_R3$, the order for *cro* protein is $O_R3 > (O_R1, O_R2)$. As predicted by these results, *cro* protein, like the λ repressor, blocks *in vitro* transcription of *cI* and *cro* from the two divergent promoters that overlap O_R. Also as predicted, transcription of *cI* is turned off at lower *cro* protein concentrations than is transcription of *cro*, whereas the opposite order of repression is obtained with λ repressor. These results describe the molecular mechanism of *cro* protein action and show that two regulatory proteins can bind to the same three adjacent sites in DNA with markedly different consequences.**

Bacteriophage λ codes for two repressor proteins. One is required for maintenance of the lysogenic state and the other for lytic phage growth. The first of these, the product of the *cI* gene, turns off transcription of the other phage genes and renders the lysogen immune to superinfection by other λ phages. This protein, known as the λ repressor, is also an autogenous regulator: at low concentrations it stimulates transcription of *cI* and at higher concentrations it represses this transcription (for review, see ref. 1). The second repressor, the product of a gene variously called *cro* or *tof*, functions midway in the lytic cycle to turn down expression of the early genes (including that of *cro* itself) and of the *cI* gene (for review, see ref. 2). Genetic experiments as well as experiments performed *in vitro* suggest that *cro* protein recognizes the same regions of DNA as does λ repressor (3–6). *cro* protein and λ repressor bind specifically to DNA bearing the λ operators and repress transcription of the *cro* operon at the operator O_R as well as transcription that originates at the second λ operator, O_L.

The mechanism of action of the λ repressor has been described in considerable detail (1). For example, repressor bound to O_R mediates both repression of the *cro* operon and autogenous regulation of *cI* (Fig. 1). The operator contains three repressor binding sites (O_R1, O_R2, and O_R3). These sites overlap two promoters P_R and P_{RM} as shown in the figure. λ repressor bound to O_R1 and O_R2 turns off transcription of the *cro* operon, which begins at P_R, and λ repressor bound to O_R3 (and perhaps O_R2) turns off transcription of *cI*, which begins at P_{RM}. In each case the λ repressor prevents binding of RNA polymerase to the corresponding promoter. Stimulation of *cI* transcription by λ repressor requires that the protein be bound to O_R1. The role of O_R2 in stimulation is not yet clear. The relative affinity of the sites for λ repressor, $O_R1 > O_R2 > O_R3$, ensures that in a lysogen, transcription of *cI* is maintained at a moderate and constant level whereas that of the *cro* operon is virtually abolished.

In this study we explored the mechanism of *cro* protein action by using two techniques. First, using the technique of Gilbert *et al.* (7), we probed the specific sites on λ DNA that are contracted by the protein by determining which bases were protected when the DNA was methylated by dimethyl sulfate in the presence of the protein. Second, we measured the effect of purified *cro* protein on transcription *in vitro* originating at P_R and P_{RM}. Our dimethyl sulfate protection experiments show that *cro* protein binds to the same three regions in O_R as does λ repressor, and that the purines protected by *cro* protein are a subset of those protected by λ repressor. These experiments also show that the order of affinity for *cro* protein is $O_R3 > (O_R1, O_R2)$. As predicted by these findings, *cro* protein blocks transcription of *cI* at lower concentrations than are required to turn off *cro* transcription. Taken together, these results provide a molecular explanation for the mechanism of action of the *cro* protein. *cro* protein first binds to O_R3 and turns off *cI* transcription by preventing binding of RNA polymerase to P_{RM}. At higher concentrations, *cro* protein then binds to O_R1 and O_R2 and also turns off transcription of *cro* by preventing binding of RNA polymerase to P_R.

MATERIALS AND METHODS

Enzymes and Reagents. Restriction endonucleases *Hae* III, *Hin*dII and III, *Alu*, *Hha* I, and *Hph* were prepared by A. Jeffrey. *Hin*f was a gift of J. Wang. *Hin*cII was purchased from New England BioLabs. Some of the polynucleotide kinase was a gift of W. McClure; the remainder was obtained from Boehringer Mannheim. RNA polymerase was obtained from R. Burgess and λ repressor was isolated by R. T. Sauer. [γ-^{32}P]ATP (3000 Ci/mmol) and [α-^{32}P]CTP (350 Ci/mmol) were purchased from New England Nuclear. *cro* protein was purified from strain 294/p*cro*1 by a method to be published elsewhere and was >90% pure as judged by sodium dodecyl sulfate gel electrophoresis. An alternate procedure for purifying *cro* protein is given in refs. 5 and 6.

Restriction Fragments and End Labeling. Fragments were purified from λ*c*I857 DNA and from pKB252 plasmid DNA (8) by the method of Maniatis *et al.* (9). End labeling with polynucleotide kinase was done as described by Maxam and Gilbert (10). Unless indicated otherwise, the strategies used to obtain specific restriction fragments bearing O_R and labeled at one end were described in detail by Humayun *et al.* (11).

Dimethyl Sulfate Protection. Fragments of duplex DNA, labeled at one end with ^{32}P, were subjected to three sequential chemical reactions: (*i*) partial methylation in the presence and absence of bound protein, (*ii*) release of methylated adenine and guanine, and (*iii*) breakage of the DNA backbone at

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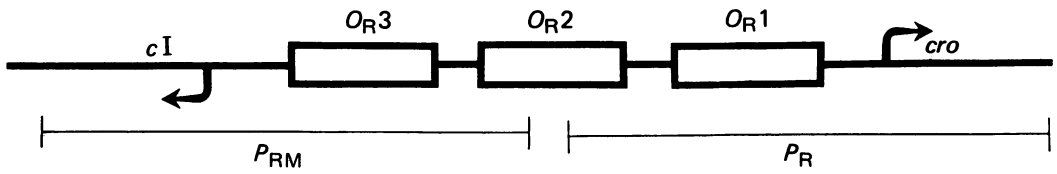

FIG. 1. Diagram of the λ O_R region. O_R1, O_R2, and O_R3 are the λ repressor binding sites, each 17 base pairs long. P_R and P_{RM} are promoters for genes *cro* and cI, respectively. The regions of DNA covered by RNA polymerase bound to each of these promoters are shown (see ref. 1). The startpoints of transcription of cI and *cro* are indicated by arrows. The DNA sequence of much of this region is shown in Fig. 3.

depurinated positions. The products of these reactions were electrophoresed through a denaturing polyacrylamide gel and autoradiographed to produce a pattern of bands. Each band corresponds to a particular purine in the sequence, and its position on the sizing gel is a measure of the distance from the ^{32}P-labeled end to that purine. The intensity of each band thus reflects the extent of methylation of a specific purine. Bound proteins have been found to suppress and, less frequently, to enhance methylation of particular purines lying in their recognition sites (7, 11–13; see also ref. 14). Conditions of methylation were as described by Gilbert *et al.* (7) with the following changes: methylation buffer contained 50 mM sodium cacodylate, pH 7.0, 10 mM $MgCl_2$, and 2.5 μg of chick blood DNA (Calbiochem) per ml methylation was performed at 0° with dimethyl sulfate at 25–100 mM for 20 min to 1 hr. *cro* protein and repressor were added at a 10- to 1000-fold molar excess over DNA. Depurination of methylated DNA with neutral phosphate (which gives dark guanine and light adenine bands), or with piperidine (which gives only guanine-specific bands) was performed by the methods of Maxam and Gilbert (10), as were strand scission, gel electrophoresis, and autoradiography.

Transcription Assays. Transcription assays were performed and the products were visualized by gel electrophoresis as described previously (15). In all cases transcription at 37° was initiated by addition of ribonucleoside triphosphates and heparin (15). Concentrations of *cro* protein, λ repressor, and RNA polymerase are given in the figure legends.

Strain. An *su*II$^+$ *pro*$^-$ derivative of 294 (8) was transformed with plasmid p*cro*1 to produce strain 294/p*cro*1. The construction and properties of p*cro*1 will be described in detail elsewhere. Briefly, p*cro*1 is a derivative of pMB9 that carries the λ *cro* gene and an O_R allele that bears mutations in O_R1 (V_s326; see ref. 16) and in O_R2 (V_{3c}; see ref. 17). The plasmid was constructed by recombination *in vitro* under conditions conforming to the standards outlined in the National Institutes of Health guidelines. About 0.02% of the soluble protein produced by 294/p*cro*1 is *cro* protein.

RESULTS

cro protein recognizes the λ repressor binding sites

Fig. 2 *a* and *b* shows the effect of *cro* protein on methylation of guanine residues in O_R1. Bases in and around the operator are numbered beginning at the *Hin*cII site as shown in Fig. 3, and the bands in Fig. 2 are numbered to indicate the corresponding G in the sequence. The experiment of Fig. 2*a* examined those guanines found in the "top strand" (Fig. 3) and shows that *cro* protein protected G +19, G +20, and G +22 from methylation; the reactivity of G +16 was markedly enhanced. No effect of *cro* protein was seen on the methylation of G +17 or any guanine to the right of O_R1. The experiment of Fig. 2*b* examined guanines found in the "bottom strand" (Fig. 3) and shows that (*i*) the reactivity of G +11 was enhanced 5-fold, (*ii*) that of G +14 was suppressed, and (*iii*) that of G +18 was not substantially affected. The effect of *cro* protein on the methylation of G +12 could not be assessed from this experiment because of the large enhancement of G +11. We have used a shorter restriction fragment (labeled at the *Hph* cleavage site, 30 base pairs to the right of the *Hin*cII cut in O_R) to show that G +12 was, in fact, protected by *cro* protein (data not shown). Fig. 2*b* also shows that, as reported (11), λ repressor protected G +18, G +14, and G +12 from methylation.

Fig. 2*c* demonstrates that *cro* protein also protects guanines in the λ repressor binding sites O_R2 and O_R3. The autoradiogram depicts the "bottom strand" of these sites and shows protection of guanines at positions −10, −11, −13, −33, −34, and −36. The experiment of Fig. 2*d* shows the effect of both *cro* protein and λ repressor on the methylation of guanines and adenines in the top strand of O_R1, O_R2, O_R3, and flanking sequences. A single restriction fragment bearing all these sites was used. As reported (11), repressor protected guanines −32, −28, −27, −26, −9, −7, −5, −3, +17, +19, +20, and +22 from dimethyl sulfate and enhanced the reactivity of G +16. *cro* protein protected and enhanced the identical set of guanines except for those at −32 (in O_R3), −9 (in O_R2), and +17 (in O_R1; see also Fig. 2*a*) which remained unaffected. The light, unnumbered bands seen in Fig. 2*d* correspond to adenines in the sequence. Neither *cro* protein nor λ repressor (as previously reported) had any noticeable effect on their methylation. Other experiments (data not shown) show that *cro* protein, like λ repressor, protected no adenines in O_R.

The results of these dimethyl sulfate protection studies are summarized in Fig. 3. The following conclusions can be drawn: (*i*) *cro* protein protects from methylation only certain guanines that lie in the λ repressor binding sites, O_R1, O_R2, and O_R3; it does not affect guanines in the spacer regions or those within about 50 bases on either side of O_R. (*ii*) *cro* protein does not block the methylation of adenine residues. (*iii*) The guanines protected from dimethyl sulfate by *cro* protein are a subset of those shielded by λ repressor. At each binding site, one or two of the λ repressor-protected guanines lying near the center of the site are not protected by *cro* protein. (*iv*) *cro* protein and λ repressor both cause substantial increases in the rates of methylation of certain guanines (for *cro* protein, G +16 and G +11; for λ repressor, G +16 only). The mechanism underlying enhancement is not known.

In addition to the major effects of *cro* protein and λ repressor on the methylation of O_R summarized in Fig. 3, we have found several minor, but reproducible, enhancement effects. Both *cro* protein and λ repressor cause slight enhancements in the reactivity of A + 23 and A − 37; *cro* protein slightly enhances the methylation of G +18; λ repressor causes a slight increase in the reactivity of G +1 and G +11.

Suggested order of binding of *cro* protein

The experiments of Fig. 2 *d* and *e* show the effects of two different concentrations of both *cro* protein and λ repressor on methylation of guanines in O_R1, O_R2, and O_R3. Careful inspection of these figures reveals that, at both concentrations,

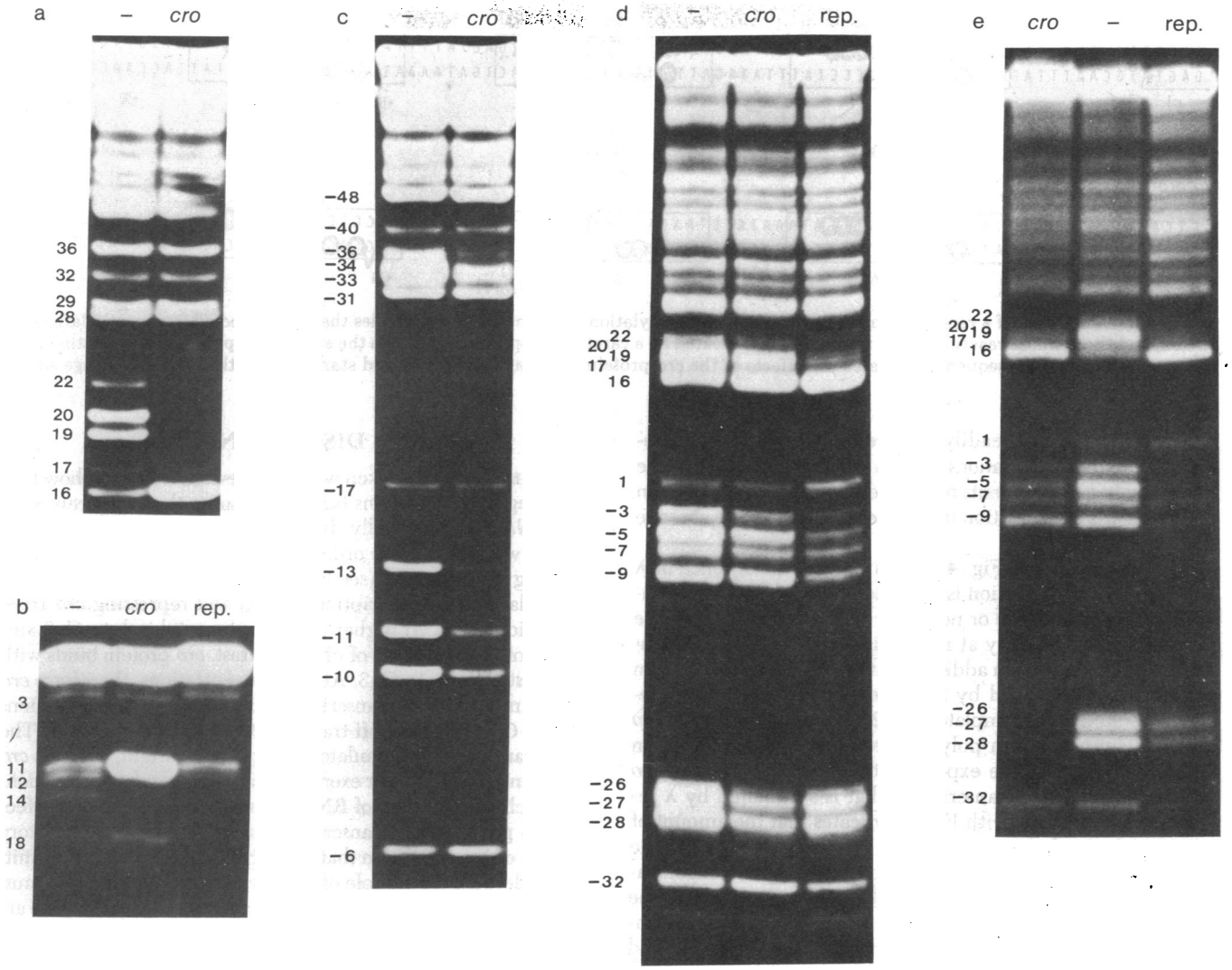

FIG. 2. Effects of *cro* protein and λ repressor on methylation of O_R. Bands corresponding to the guanines in and around O_R are numbered according to Fig. 3. The values given below for molar excesses of *cro* protein and λ repressor are only approximate. (*a*) "Top strand" of O_R1 (see Fig. 3). The *Hin*cII/*Hae*III 140 restriction fragment (see ref. 11) labeled at the *Hin*cII end in O_R was methylated in the absence (lane 1) and presence (lane 2) of a 50-fold molar excess of *cro* protein. Methylated DNA was depurinated using neutral phosphate. (*b*) "Bottom strand" of O_R1. A fragment of DNA labeled at the *Hha* I end position +87 (see Fig. 3) and extending to the *Hin*cII cut in O_R was methylated in the absence (lane 1) and presence of a 50-fold excess of *cro* protein (lane 2) and λ repressor (lane 3). Methylated DNA was treated as in *a*. (*c*) "Bottom strand" of O_R2 and O_R3. A restriction fragment labeled at the *Hin*cII end in O_R and extending to a *Hin*f cut at position −153 was methylated in the absence (lane 1) and presence (lane 2) of a 200-fold molar excess of *cro* protein. Methylated DNA was treated as in *a*. (*d*) "Top strand" of O_R3, O_R2, and O_R1. The *Alu*/*Hha* I 160 fragment (see ref. 11) labeled at the *Alu* end was methylated in the absence (lane 1) and presence of a 50-fold molar excess of *cro* protein (lane 2) and λ repressor (lane 3). Reacted DNA was treated as in *a*. (*e*) "Top strand" of O_R3, O_R2, and O_R1. The *Alu*/*Hha* I 160 fragment (see *d*) was methylated in the absence (lane 2) and presence of 1000-fold molar excess of *cro* protein (lane 1) and λ repressor (lane 3). Piperidine was used for depurination.

λ repressor protected guanines in O_R1 more completely than those in O_R3, and that guanines in O_R2 are protected as efficiently as those in O_R1 only at the higher λ repressor concentration (Fig. 2*e*). This result, as reported previously (11), is consistent with the order of affinity for λ repressor $O_R1 > O_R2 > O_R3$ determined by different experiments (1). In contrast are the results with *cro* protein. At both low (Fig. 2*d*) and high (Fig. 2*e*) *cro* protein concentrations, O_R3 was protected more completely than were O_R1 and O_R2. In the experiment of Fig. 2*d*, O_R1 was protected more completely than was O_R2, whereas in Fig. 2*e* they were protected about equally. Assuming that protection is a measure of occupancy, these results show that, in contrast to λ repressor, *cro* protein binds with an affinity order $O_R3 > (O_R2, O_R1)$, and that perhaps $O_R1 > O_R2$.

Effect of *cro* protein on transcription from P_R and P_{RM} *in vitro*

The finding that *cro* protein binds to O_R3 more tightly than to O_R1 predicts that a lower concentration of *cro* protein is required to turn off *c*I transcription than is required to turn off *cro* transcription. This follows from the relationship of the promoters to the λ repressor binding sites as shown in Fig. 1 and from the assumption that *cro* protein, like λ repressor, blocks transcription by excluding binding of RNA polymerase.

Transcription of *c*I from P_{RM} and *cro* from P_R can be assayed *in vitro* by using, as a template, a single DNA fragment bearing these promoters and flanking sequences. Appropriate amounts of λ repressor stimulate *c*I and repress *cro* transcription.

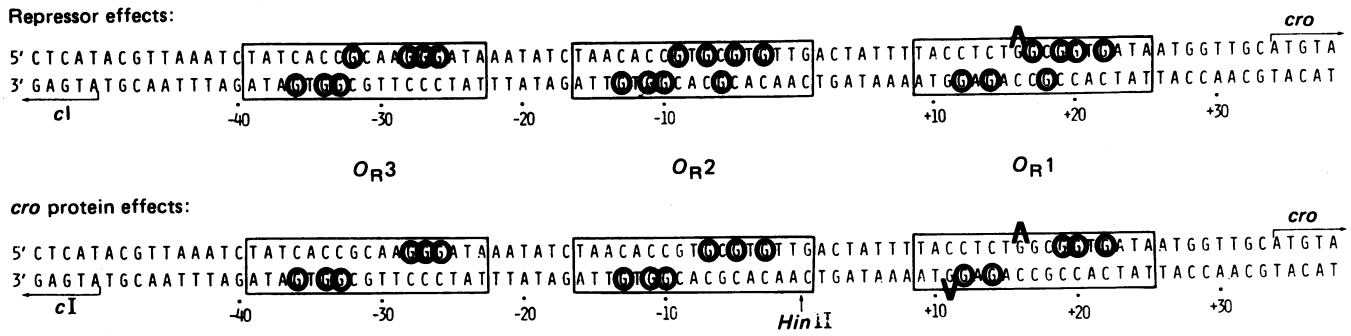

FIG. 3. The effects of λ repressor and *cro* protein on the methylation of purines in O_R. Guanines that are protected from methylation are circled, and those whose reaction rate is increased are indicated by a caret. The top sequence shows the effects of repressor on the methylation of O_R (11). The bottom sequence indicates the effects of the *cro* protein. The bases are numbered starting from the *Hin*cII cleavage site in O_R.

Transcription of *c*I is readily observed in the absence of λ repressor if high concentrations of RNA polymerase are used. The following two experiments measured the effect of *cro* protein on *c*I and *cro* transcription in the presence and in the absence of λ repressor.

The experiment of Fig. 4 reveals that, in the absence of λ repressor, *c*I transcription is abolished at *cro* protein concentrations that have little or no effect on *cro* transcription. The latter is turned off only at much higher *cro* protein concentrations. Fig. 4 shows in addition that *cro* protein does not turn off transcripts directed by RNA polymerase molecules previously bound to the template (lane 2). This is as expected if *cro* protein competes with polymerase for overlapping sites on DNA (see Fig. 1). The experiment of Fig. 5 shows that *cro* protein turns off *c*I transcription that is stimulated by λ repressor. Comparison with Fig. 4 indicates that the amount of *cro* protein needed to repress this *c*I transcription is the same as that needed in the absence of repressor. Higher concentrations of *cro* protein turn off residual *cro* transcription as in the experiment of Fig. 4. Fig. 5 also shows an unexpected enhancement of *cro* transcription by *cro* protein (see lanes 3 and 4 of Fig. 5.) The stimulatory effect of *cro* protein on *cro* transcription has also been observed in the absence of λ repressor when low RNA polymerase concentrations were used. Both the negative and positive effects of *cro* protein that we have observed are specific: *cro* protein had no effect on transcription originating at the *lac* promoter bearing the UV5 mutation (18) (data not shown).

DISCUSSION

Our current results, taken with our previous work (1), show that two regulatory proteins bind to the same three adjacent sites in DNA with markedly different consequences. λ repressor binds with an affinity order $O_R1 > O_R2 > O_R3$ with the following consequences (see Fig. 1): repressor first binds to O_R1, stimulating *c*I transcription at P_{RM} and repressing *cro* transcription at P_R. At higher concentrations it binds to O_R3 and turns off transcription of *c*I. In contrast, *cro* protein binds with highest affinity to O_R3. At low concentrations, therefore, *cro* protein turns off *c*I transcription, and at higher concentrations it fills O_R1 and turns off transcription of its own gene, *cro*. The mechanism of the stimulatory effects is uncertain, but both *cro* protein and λ repressor exert their negative regulatory functions by excluding binding of RNA polymerase. The negative effect of *cro* protein on *c*I transcription is epistatic to the stimulatory effect of λ repressor on that transcription. This summary omits consideration of the role of O_R2; we know that repressor must be bound to O_R2 for complete repression of P_R (15), but we are uncertain of the role of O_R2 in the other regulatory functions of λ repressor and *cro* protein. Our current experiments also suggest the possibility that *cro* protein might be a positive regulator of transcription of its own gene. *In vitro*, low concentrations of *cro* protein specifically enhance transcription beginning at P_R, presumably by binding to O_R3. An assessment of the physiological significance of this finding will require further experimentation.

It is remarkable that two proteins with amino acid sequences

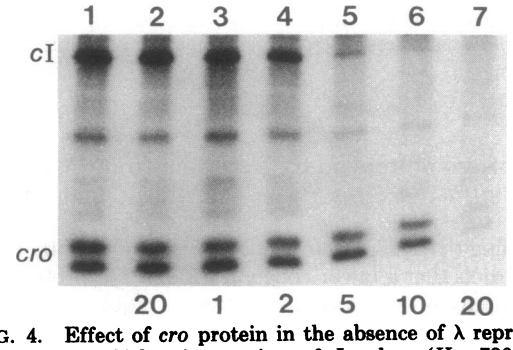

FIG. 4. Effect of *cro* protein in the absence of λ repressor. A fragment of λ DNA bearing portions of *c*I and *cro* (*Hae* 790, ref. 15) was transcribed in seven separate reactions by a 20-fold molar excess of RNA polymerase. Transcripts of *c*I and *cro* were identified previously (15). The *cro* transcript appears as a double band, the smaller being a product of premature termination. Lane 1, no *cro* protein; lane 2, *cro* protein added following addition of RNA polymerase; lanes 3–7 *cro* protein added before addition of RNA polymerase. The relative amount of *cro* protein added is shown at the bottom.

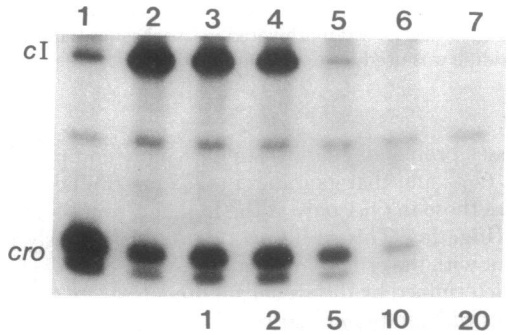

FIG. 5. Effect of *cro* protein in the presence of λ repressor. The template used in the experiment of Fig. 4 was transcribed in seven separate reactions by a 4-fold molar excess of RNA polymerase. Lane 1, no repressor and no *cro* protein; lane 2, sufficient λ repressor present to stimulate *c*I transcription about 10-fold, no *cro* protein; lanes 3–7, as in lane 2 plus increasing amounts of *cro* protein, shown in relative amounts. In each case the order of addition was repressor, then *cro* protein, then RNA polymerase.

exhibiting no obvious homologies (19–21) bind to the same three regions of DNA within O_R. *cro* protein is a highly basic protein consisting of 66 amino acids, and λ repressor is a weakly acidic protein of 236 amino acids. *cro* protein is isolated as a stable dimer which is apparently the DNA binding form, whereas λ repressor monomers are in concentration-dependent equilibrium with dimers which are the DNA binding form. The affinity of *cro* protein for DNA, as measured by using nitrocellulose filters, is two to three orders of magnitude below that of λ repressor ($K_d \simeq 10^{-13}$M) (see refs. 6, 22).

Our dimethyl sulfate protection experiments have thus far probed only guanine and adenine contacts and have revealed that both λ repressor and *cro* protein protect guanines but not adenines from methylation. This suggests that both proteins contact DNA primarily in the major groove. This follows from the fact that the sites of methylation we have probed, N^7 on guanine and N^3 on adenine are exposed in the major and minor grooves, respectively (see ref. 12). *cro* protein protects two fewer guanines in O_R1 and O_R2 and one fewer guanine in O_R3 than does λ repressor. These differences are consistent with the different order of affinities of the two proteins for the three sites.

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