Proc. Nat. Acad. Sci. USA
Vol. 72, No. 12, pp. 4785–4789, December 1975
Biochemistry

λ Repressor turns off transcription of its own gene

(autogenous control/restriction endonuclease fragment/DNA sequence/promoter mutation/transcription)

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Communicated by Matthew Meselson, September 10, 1975

ABSTRACT **We report transcription *in vitro* of the λ repressor gene, *cI*, using specific restriction endonuclease fragments as templates. This transcription is repressed by λ repressor. Moreover, we report the sequence change caused by a *cI* promoter mutation. This change is located between two repressor binding sites in the rightward operator (O_R). Transcription studies using mutant templates indicate that repressor bound to two sites in O_R regulates transcription of gene *tof*, and repressor bound to the remaining site(s) controls transcription of *cI*.**

The λ repressor binds to two operators on λ DNA, O_L and O_R, thereby blocking rightward transcription of gene *tof* (sometimes called *cro*) and leftward transcription of gene *N* (see Fig. 1 and for review see ref. 1). The operators contain multiple repressor binding sites (2, 3). In the sequence of each operator there are three closely related 17 base pair sequences, separated by A+T-rich spacers, which we have identified as repressor binding sites (4).* Transcripts of *N* and *tof* begin just outside each adjacent operator (5), and in each case an RNA polymerase binding site overlaps a repressor binding site (6, 4). Fig. 2 shows the sequence of O_R, including three repressor binding sites and various restriction endonuclease cleavage sites.

Gene *cI*, which encodes the repressor, is located between these operators. In a lysogen transcription of *cI* begins near the right operator and proceeds leftward (7, 8). Experiments performed *in vivo* suggest that repressor controls its own synthesis both negatively (9) and positively (for example, see ref. 10). Although repressor has been synthesized in a crude system *in vitro* (11), transcription of *cI* using purified components has not been reported heretofore.

We report in this paper transcription of *cI* *in vitro* using purified RNA polymerase and fragments of λ DNA isolated using restriction endonucleases. We find that this transcription is repressed by λ repressor. Moreover, we report that the mutation *P*rm116, characterized as a *cI* promoter mutation (3), abolishes *cI* transcription *in vitro* and is located between two repressor binding sites in O_R. These and other results indicate that a repressor binding site in O_R overlaps sequences that must be recognized by polymerase for *cI* transcription. Further analysis indicates different roles for the three repressor binding sites in O_R: repressor bound to the rightmost sites, O_R1 and O_R2, regulates *tof* transcription, whereas repressor bound to O_R3 controls transcription of its own gene, *cI*.

MATERIALS AND METHODS

Enzymes and Reagents. Restriction endonucleases *Hae* III (from *Haemophilus aegyptius*) *Hin*d(II & III) (from *H. influenzae* d) and *Hph* (from *H. parahaemolyticus*), gifts from A. Jeffrey and Z. Humayun, were prepared as described (12). In the text the enzyme *Hin* refers to a mixture of *Hin*d(II & III). λ Repressor was a gift from P. Chadwick and was assayed as described (13). RNA polymerase was prepared according to the procedure of Berg *et al.* (14). Molarity of the polymerase is based on total protein, and the exact specific activity is not known. α-^{32}P-Labeled ribonucleoside triphosphates (100–120 Ci/mmol) were obtained from New England Nuclear.

Restriction Endonuclease Fragments. Fragments were prepared from phage DNA as described by Maniatis *et al.* (12).

Polyacrylamide Gel Electrophoresis. Five percent acrylamide–0.16% bis-acrylamide–7 M urea polyacrylamide gels were prepared and transcript sizes were estimated according to Maniatis *et al.* (15).

Phage Strains. The following phage strains were obtained from Stuart Flashman and were grown by liquid infection: λ*v*101*v*1*v*3S7, λ*v*002v_NS7, λ*vs*326*c*II68S7, λ*P*rm116*c*II68S7. The mutations *vs*326 (16), v_N (Flashman, unpublished), and *v*1*v*3 (17) decrease the affinity of O_R for repressor both *in vivo* and *in vitro*.

Transcription Assays. Twenty microliter reactions contained the following: 0.04 M Tris·HCl pH 8.0, 0.01 M $MgCl_2$, 0.05 M KCl, 10% glycerol, 0.01 M 2-mercaptoethanol, 10 nM DNA fragments, 5–80 neq/liter of repressor, 40–200 nM RNA polymerase. Unlabeled ATP was used at 250 μM and heparin (Upjohn Co.) at 100 μg/ml. One triphosphate (either [α-^{32}P]CTP, [α-^{32}P]GTP, or [α-^{32}P]UTP) was about 2.5 μM and the other two, unlabeled, were at 5 μM. DNA fragments were incubated with and without repressor for 10 min at 37° followed by incubation with RNA polymerase for an additional 10 min. NTP's and heparin were then added simultaneously and the mixtures were incubated 15 min at 37°. Reactions were stopped by addition of 20 μl deionized formamide, and samples were loaded directly onto the gels. Transcripts were quantitated by excising the gel bands, suspending them in 5 ml of H_2O and counting Cerenkov radiation. In some experiments RNA polymerase was added first and followed 10 min later by repressor; reactions were then begun after a further 10 min incubation by the addition of the NTP's and heparin.

RNA·DNA Hybridization. Hybrids made according to Roberts (18) were collected by diluting 200 μl samples to 3

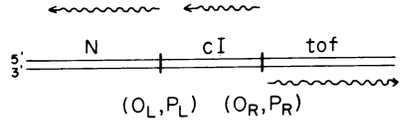

FIG. 1. Schematic map of a portion of the λ genome. Directions of transcription of genes *N*, *cI*, and *tof* are indicated with wavy arrows. The leftward and rightward operator-promoters are indicated.

Abbreviations: bp, base pairs; WT, wild type.

* For a discussion of the relationship between these sequences and the fragments protected from nuclease digestion by repressor see ref. 4.

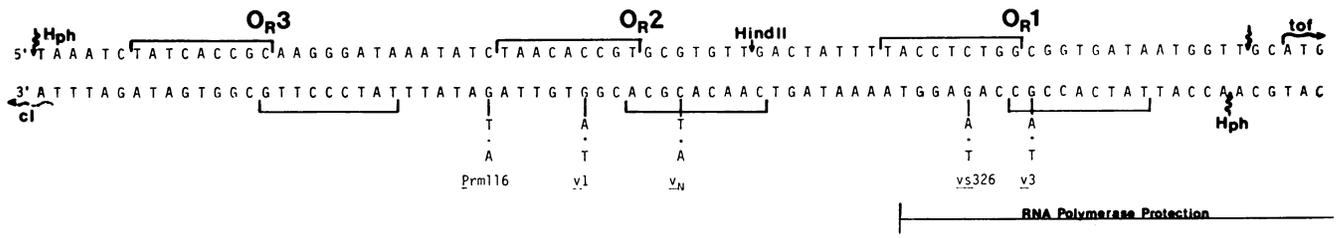

FIG. 2. Sequence of O_R. Three presumed repressor binding sites, O_R1, O_R2, and O_R3, are set off by brackets. The base changes caused by one promoter mutation (*Prm116*) and by four operator mutations are indicated. The location of the operator constitutive mutations was deduced by a combination of repressor binding studies and DNA sequence analyses (refs. 4 and 23; S. Flashman, D. Kleid, and M. Ptashne, in preparation). The portion of DNA protected by RNA polymerase from DNase digestion is indicated for the *tof* promoter (6). The start-points of transcription of *tof* and the approximate location of that of *cI* are indicated by arrows. The positions of *Hin* and *Hph* cleavage sites are shown (3, 12).

ml with filtering buffer (0.01 M Tris·HCl, pH 7.5, 0.5 M KCl) and filtering slowly through Schleicher and Schuell B-6 membranes presoaked in buffer. Membranes were washed with 40 ml of buffer.

RESULTS

Transcription of *cI in vitro*

The RNA transcripts produced *in vitro* from a DNA molecule containing parts of *cI* and *tof* were analyzed by polyacrylamide gel electrophoresis and are shown in Fig. 4. The template (*Hae* 790) was a 790 base pair (bp) fragment produced by cleavage of λ DNA with the restriction endonuclease *Hae* III (see Fig. 3). No repressor was present in these experiments. Fig. 4 shows that three prominent transcripts were produced: two are about 110 bases long, the third about 300 bases long. The shorter species were identified as *tof* transcripts and the longer as the *cI* transcript by the following criteria:

(*1*) the "*cI* transcript" was produced in greatly decreased amounts if the template carried the mutation *Prm116*, whereas the yield of the "*tof* transcripts" was unchanged;

(*2*) the "*cI* and *tof* transcripts" hybridized, respectively, with a 5-fold or greater specificity to the *l* and *r* strands of λ DNA (not shown);

(*3*) the *Hae* 790 cleaved with *Hin* did not direct synthesis of the "*tof* transcript," but did direct synthesis of the "*cI* transcript." Previous experiments have shown that the polymerase recognition site required for transcription of *tof* includes bases in the *Hin*dII site in O_R2 (19) (see Fig. 2). Moreover, the full length "*cI* transcript" from the *Hin*-cleaved *Hae* 790 was shorter than that from the *Hae* 790, as expected (see Fig. 3);

(*4*) operator constitutive mutants that decrease the effect of repressor on *tof* expression *in vivo* have a similar effect on the "*tof* transcript" *in vitro* (see Fig. 6).

The *cI* transcript seen in Fig. 4 does not extend to the end of the template. In the experiment of that figure, UTP was used at 2.5 μM. In other experiments (see for example that of Fig. 6) in which the UTP concentration was higher, longer transcripts were observed, including one corresponding to the expected length for fully extended transcripts. In all experiments presented here the two *tof* transcripts were produced coordinately, and we believe one to be an extension of the other. In the presence of 5 μM UTP only the longer *tof* transcript was seen. Premature termination of transcription, producing "stutter products," has been observed by others (e.g., ref. 20).

Transcription of *cI* is much more sensitive to increasing ionic strength than that of *tof*. At 50 mM KCl, and with RNA polymerase in a 20-fold molar excess, the ratio of *cI* to *tof* initiations was about 1:3. At 150 mM KCl *cI* transcription was virtually abolished, whereas *tof* transcription was reduced no more than 2-fold. When glycerol was omitted from the reaction with wild-type template, the ratio of *cI* to *tof* initiations was about 1:6 (not shown). Glycerol did not reverse the block to *cI* transcription *in vitro* from DNA bearing *Prm116*.

From the location of the mutation *Prm116* (see Figs. 2 and 7) we surmised that transcription of *cI* originates in or very near O_R. This conclusion was also reached by the following argument. Cleavage of *Hae* 790 with *Hin* yields a 375 bp fragment (*Hin* 375) (see Fig. 3) that is an efficient template for *cI* transcription. The right terminus of *Hin* 375 is within a repressor binding site (O_R2) in O_R (Fig. 2). Moreover, *Hin* 375 contains a site cut by the restriction enzyme *Hph* located 45 bp from the right end (see Fig. 2). Cleavage of this fragment with *Hph* destroyed its ability to direct *cI* transcription.

Repressor turns off transcription of *cI*

Fig. 5 shows the effect of increasing repressor concentrations on transcription directed by the *Hae* 790 template. Repressor blocked transcription of *tof*, as reported previously (21), and, at somewhat higher concentrations, blocked transcription of *cI*. We believe this to be a specific effect of repressor because, as shown in the experiment of Fig. 6, repressor concentrations sufficient to decrease *cI* transcription 10-fold had no effect on *tof* transcription if the template bore mutations in the operator sites controlling *tof* transcription (see Fig. 6 and *Discussion* below). This argues that our repressor preparation does not contain some nonspecific inhibitor of transcription that is apparent only in the concentration range needed to repress *cI* transcription. Moreover, our sequence information (see below) suggests that the *cI* promoter overlaps a repressor binding site in O_R, O_R3, just as the *tof* promoter overlaps a different repressor binding site in O_R, O_R1. The fact that our repressor preparation blocks *tof* and *cI* transcription only if added to the template before addition of RNA polymerase (not shown) is consistent with our assumption that the active inhibitor in both cases is repressor.

Different functions of the repressor binding sites in O_R

The following observations indicate that efficient repression of *tof* transcription *in vitro* requires binding of repressor to both O_R1 and O_R2, whereas *cI* transcription is efficiently repressed by repressor bound to O_R3. Transcription of *tof* from a template bearing operator constitutive mutations in both O_R1 and O_R2 (*v1v3*) was resistant to high concentra-

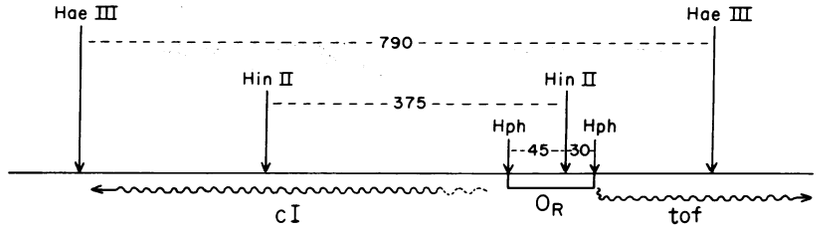

FIG. 3. Schematic portion of the λ genome showing positions of various endonuclease cleavage sites (24, 3, 12). Approximate distances in base pairs between sites are shown. The distance from the startpoint of *tof* transcription to the end of the *Hae* 790 fragment is approximately 110 bp.

tions of repressor. Repression of *c*I transcription, however, was about the same as that observed with a wild-type template (Fig. 6). In experiments not shown, we measured the effect of repressor using the templates singly mutated in either O_R1 (*vs*326) or O_R2 (v_N) (see Fig. 2), and found that the sensitivity of *tof* transcription to repression was between that of the wild-type and *v*1*v*3 templates. Repression of *tof* transcription was less efficient with the v_N than with the *vs*326 template. In both cases, however, repression of *c*I transcription was observed. Moreover, transcription of *c*I from *Hin* 375, which contains an intact O_R3, part of O_R2, and none of O_R1, was repressed as efficiently as that directed by a template containing an intact O_R (not shown).

Does repressor enhance transcription of *c*I?

An examination of Fig. 5 reveals that low concentrations of repressor increased 3-fold the level of *c*I transcription. The experiment was performed with a ratio of polymerase to DNA molecules of roughly four to one, and in experiments not shown, we observed the amount of repressor stimulation of the *c*I transcript to be dependent upon the concentration of polymerase in each reaction. We do not know the mechanism of this stimulation, nor do we know its relevance to control *in vivo*.

The sequence change of *P*rm116

*P*rm116 replaces a G·C with an A·T in the spacer between O_R2 and O_R3 (Fig. 2). This change was determined by direct DNA sequence analysis. A detailed description of these methods has been presented elsewhere (12). The argument in outline is as follows: The combined action of *Hph* and *Hin* produces a 45 bp fragment (*Hin*/*Hph* 45) that contains O_R3 and most of O_R2 (Fig. 2). Analysis of the partial exonuclease digestion products of this fragment, labeled with ^{32}P at either end, tentatively identified the substitution of an A for a G at the position 17 nucleotides to the left of the *Hin* end on the *r* strand (Fig. 7). This was confirmed by pyrimidine tract analysis: the *l* strand of *Hin*/*Hph* 45 bearing *P*rm116 contains a T_3 with 3′ nearest neighbor A, whereas wild type does not, and it contains one less T_2C with 3′ nearest neighbor A than does wild type (Table 1). All the other pyrimidine tracts in *Hin*/*Hph* 45 and in *Hin*/*Hph* 30 which contains 30 bp to the right of the *Hin* site in O_R (see Fig. 2), were unaffected by *P*rm116. The sequence change was further confirmed by an independent method for sequencing mutant changes in DNA using dimethyl sulfate (22) (not shown).

The sequence change we report for *P*rm116 is within the five base-pair region identified by Smith *et al.* to contain this mutation (unpublished manuscript). Their methods suggest a different sequence change for *P*rm116, but they have not determined an exact change.

DISCUSSION

We have presented two independent lines of evidence that suggest a mechanism for how repressor turns off transcription of its own gene, *c*I. *First*, the sequence change caused by the mutation *P*rm116 has been located between two repressor binding sites, O_R2 and O_R3. *In vitro*, transcription of *c*I from templates bearing this mutation is greatly decreased compared to transcription from wild-type templates, confirming the surmise (3) that this mutation damages the *c*I promoter. Thus the *c*I promoter probably overlaps O_R3, just as the *tof* promoter overlaps O_R1 (4, 6, 19). Consistent with this conclusion is our finding that repression of both *tof* and *c*I does not occur if RNA polymerase is bound to the template before addition of repressor. *Second*, mutations in O_R1 and/or O_R2 that decrease the affinity of these sites for repressor do not drastically affect repression of *c*I transcription. These two lines of evidence indicate that the third repressor binding site, O_R3, mediates control of *c*I transcription. A phage mutant in O_R3 has not been isolated, and we cannot exclude the possibility that repressor bound only to O_R2 would partially repress *c*I transcription. It is also possible that O_R contains one or more additional repressor binding sites to the left of O_R3 that participate in repression of *c*I transcription.

Our results also indicate that two repressor binding sites in O_R, O_R1 and O_R2, are required for efficient control of *tof* transcription. Mutation of either of these sites decreases the

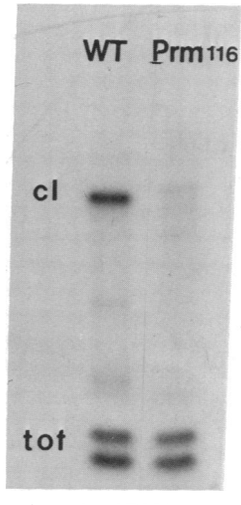

FIG. 4. Gel electrophoresis of RNA transcripts read from wild-type (WT) and *P*rm116-containing templates. The experiment was performed in the absence of repressor and with a 20-fold molar excess of RNA polymerase to DNA. The template is the restriction endonuclease fragment *Hae* 790 (see *text*). The identities of the transcripts are indicated. [α-^{32}P]UTP was used at 2.5 μM. Electrophoresis was from top to bottom.

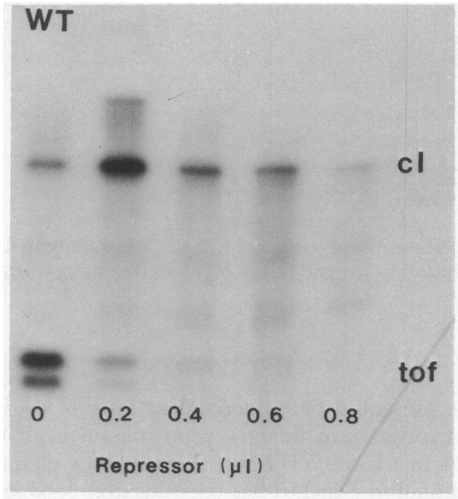

FIG. 5. The effect of increasing concentrations of repressor on *c*I and *tof* transcription from a wild-type template. The amount of *c*I transcription in the absence of repressor is shown in the first slot. A 3-fold stimulation of the *c*I transcript occurs at the lowest repressor concentration used (second slot). Repressor decreased the *c*I transcript to 40% its level in the absence of repressor (fifth slot). The molar ratio of polymerase to DNA was four to one. Aliquots of repressor were added from a repressor solution approximately 0.4 μeq/liter. The concentration of [α-^{32}P]UTP was 2.5 μM.

effect of repressor on *tof* transcription *in vitro*, and mutation of both sites has a more drastic effect. The role of O_R1 and O_R2 in mediating repressor control of *tof* transcription has been deduced by Stuart Flashman in this lab on the basis of studies of the effects of operator mutants *in vivo* and will be discussed elsewhere (in preparation). These results provide at least a partial explanation for the function of reiterated repressor binding sites in the rightward λ operator.

Our sequence results indicate that two mutations in λ DNA (*sex*1 [in preparation] and *P*rm116) cause similar

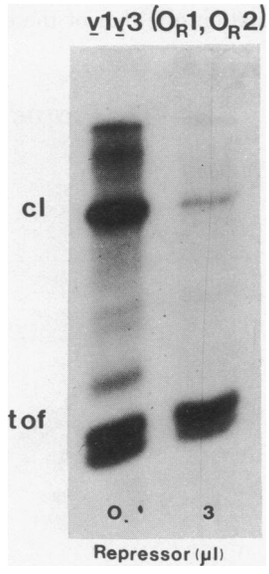

FIG. 6. Effect of repressor on *c*I and *tof* transcription from a template bearing the mutation *v*1*v*3. Repressor decreased the *c*I transcript to 10% its level in the absence of repressor. Repressor is as in Fig. 5; polymerase is in a 20-fold excess. [α-^{32}P]UTP was at about 3.5 μM, and *c*I transcripts longer than that seen in the experiments of Figs. 4 and 5 are evident (see *text*).

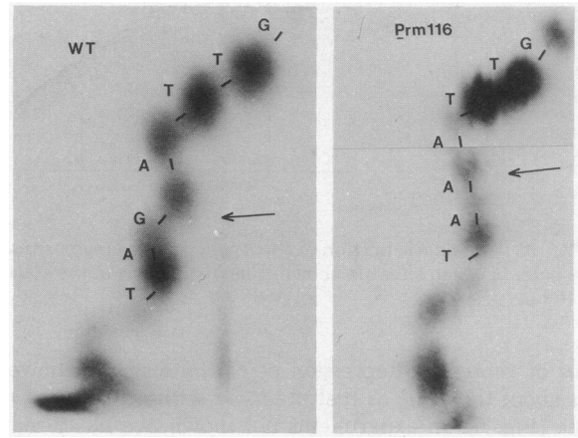

FIG. 7. Two-dimensional fractionation of partial nuclease digestion products of wild type (left) and *P*rm116 DNA (right). In each case *Hin/Hph* 45 was labeled *in vitro* with ^{32}P at the *Hin* end, digested with nuclease, and fractionated as previously described (12). The figure shows the identity of bases at positions 13–19 counting from the *Hin* end. The bases at positions 1–12 appeared identical in *P*rm116 and wild type and are not shown in the figure. An arrow indicates the sequence change caused by *P*rm116.

changes: each changes the sole G·C to an A·T in an A+T-rich spacer between repressor binding sites. The mutation *sex*1, which decreases transcription of gene *N*, is located 31 bases from the startpoint of transcription (in preparation). If there is a similar relationship between *P*rm116 and the startpoint of transcription of *c*I, then *c*I transcription begins just to the left of O_R3, and O_R2 is located midway between the startpoints of transcription of *tof* and *c*I. The fact that the two mutations in O_R2 we have studied affect *tof* repression more severely than *c*I repression suggests that the details of repression may differ in the two cases.

We have noticed that small amounts of repressor enhance *c*I transcription in experiments with limiting polymerase, but we do not know the mechanism of the effect and we do not know its relevance to the observation (10) that repressor

Table 1. Pyrimidine tracts from *Hin/Hph* 45 wild type and *P*rm116 DNA with 3′ nearest neighbor A

	Quantitation*			
	Predicted (molar ratio)		Observed (molar ratio)	
	WT	*P*rm116	WT	*P*rm116
l strand				
C	2	2	2.34	2.27
TC	1	1	0.85	1.02
T_2C	2	1	1.85	0.92
T	3	3	2.94	2.77
T_3	0	1	0	1.04
r strand				
C	2	2	1.95	2.18
T	2	2	2.22	1.84
T_2	1	1	1.05	0.98
T_3	2	2	1.77	1.99

* Predicted values for each tract based on the sequence change suggested by the experiment of Fig. 7 are given in molar ratios. In each case the restriction endonuclease fragment was labeled with [α-^{32}P]dATP *in vitro*, and the pyrimidine tracts of the separated strands were determined as described (12).

positively controls its own synthesis *in vivo*. We do not know, for example, whether polymerase binding to the *tof* and *cI* promoters is mutually exclusive. Our results do constitute a clear demonstration that repressor negatively controls its own transcription and provide a mechanism for that autogenous regulation.

We thank Gerry Smith for communicating his sequence results and Stuart Flashman for communicating other results prior to publication, and Andrea Jeffrey for technical assistance. B.M. is a predoctoral trainee and D.K. a postdoctoral fellow of the National Institutes of Health. This work was also supported by grants from the National Science Foundation and the National Institutes of Health to M.P.

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