Studies on the Mechanism of Action of the Gal Repressor

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SUMMARY

The mechanism of action of Escherichia coli gal repressor on in vitro gal messenger RNA synthesis has been studied. The purified transcription system includes RNA polymerase holoenzyme, cyclic adenosine 3':5'-monophosphate (cyclic AMP), cyclic AMP receptor protein (CRP), and a λgal DNA template. We find that gal repressor acts in a manner competitive with RNA polymerase plus CRP. A preinitiation complex formed by λgal DNA, RNA polymerase, CRP, and cyclic AMP is resistant to inhibition by gal repressor. Conversely, incubation of λgal DNA with gal repressor prior to the addition of RNA polymerase, CRP, and cyclic AMP prevents the formation of a preinitiation complex. We conclude that the gal operator and promoter regions overlap functionally, and we consider a variety of models to explain this effect.

The expression of the gal operon of Escherichia coli is under both positive and negative control. The former consists of a requirement for cyclic adenosine 3':5'-monophosphate and cyclic AMP receptor protein, which act together with RNA polymerase to form a preinitiation complex at the gal promoter (1-4). Negative control is exerted by the gal repressor protein (5-9). Repression of the gal operon is overcome by the addition of inducers such as D-fucose or D-galactose. Parks et al. (10) partially purified the gal repressor from extracts of E. coli and showed that it binds specifically to gal DNA. This binding is competitive with RNA polymerase plus CRP for binding at the gal operator-promoter region. Conversely, incubation of λgal DNA with gal repressor prior to the addition of RNA polymerase, CRP, and cyclic AMP prevents the formation of a preinitiation complex. We conclude that the gal operator and promoter regions overlap functionally, and we consider a variety of models to explain this effect.

in vitro transcription system and two inhibitors of unbound RNA polymerase, rifampin (12, 13) and the synthetic polynucleotide, poly(rI) (14, 15), to elucidate the mechanism of action of gal repressor. We find that a preinitiation complex will not form when repressor is prebound to gal DNA. Conversely, gal repressor does not inhibit transcription once a preinitiation complex has formed at the gal promoter. We interpret our results as indicating that the gal repressor competes with RNA polymerase plus CRP for binding at the gal operator-promoter region.

MATERIALS AND METHODS

Chemicals—Cyclic AMP, [5-3H]CTP (16.7 Ci per mmole), and D-fucose (6-deoxy-D-galactose) were purchased from Schwarz-Mann; UTP, ATP, GTP, and CTP from P-L Biochemicals; polynosinic acid from Miles Laboratories; Rifampin from Ciba Pharmaceutical Company; and Bio Gel P 150 (100 to 200 mesh) from Bio-Rad Laboratories.

Bacterial and Bacteriophage Strains Used—Gal repressor was purified after phage induction of the strain SA1108: F-lysA-argH- (λc1857Δ7glyA+galR+) (gift of Dr. C. Hill, Department of Biochemistry, Hershey Medical Center, Hershey, Pa.). The prophage is deleted for the λ late genes A through J. Induction of this prophage produces a high level of gal repressor. The conditions under which the lysogen is grown have been described previously (11). The bacteriophage λPG/8c1857Δ7 has also been described previously (4).

CRP, RNA Polymerase, Galactose Repressor, and Phage DNA—CRP and E. coli RNA polymerase were purified as described previously (11). Phage DNA was extracted as described by Nissley et al. (4).

Galactose repressor was purified as previously described (11), with the following modifications. All buffers contained 0.1 mM EDTA, 0.2 mM dithiothreitol, and 15% (v/v) glycerol, unless otherwise noted. After DEAE-cellulose chromatography, the pooled fractions were precipitated with addition of solid ammonium sulfate (472 mg for each ml of fraction). The resulting precipitate was dissolved in 0.1 M potassium phosphate, pH 6.0, and dialyzed against 250 volumes of the same buffer for 4 hours. The repressor preparation was then applied to a Bio Gel P 150 column (1.6 × 55 cm) equilibrated with 0.1 M potassium phosphate, pH 6.5. The column was eluted with the same buffer at a rate of 5 ml per hour. Fractions exhibiting high repressor activity were pooled and dialyzed against 50 volumes of 10 mM Tris-HCl, pH 7.5, containing 10 mM MgCl2. The gal repressor was further purified by chromatography on a 2-aminophenyl-
β-D-thiogalactoside-substituted agarose column as described previously (11). In order to concentrate the purified gal repressor, the preparation pooled after affinity chromatography was dialyzed against 50 volumes of 20 mM Tris-HCl, pH 7.5, and then applied to DEAE-cellulose equilibrated with the same buffer and eluted with 20 mM Tris-HCl, pH 7.5, containing 0.5 mM KCl. The eluate was dialyzed against 20 mM Tris-HCl (pH 7.5) containing 0.1 mM EDTA, 0.2 mM dithiothreitol, and 20% (v/v) glycerol and stored at -70°.

Reaction Assays and Detection of Labeled RNA Product—Typical reaction mixtures (0.1 ml) for the formation of preinitiation complexes contained 20 mM Tris-HCl (pH 7.9); 100 mM KCl, 0.1 mM dithiothreitol; 0.15 mM ATP, UTP, and GTP; 0.075 mM [3H]CTP (16.7 Ci per mmole); 100 pg of bovine serum albumin per ml; 5% (v/v) glycerol; 8.4 µg of λg<sub>gal</sub> DNA per ml; 20 µg of CRP per ml; 0.1 mM cyclic AMP (if required); 24 µg of RNA polymerase per ml; and 4 µM EDTA (to chelate all of the Mg<sup>2+</sup> present). The reaction mixture was incubated at 37° to form a preinitiation complex. Transcript ion was initiated by the addition of ribonucleoside triphosphates or Mg<sup>2+</sup> to a complex made in the absence of one of these components. RNA polymerase bound to DNA as a preinitiation complex is relatively resistant to rifampin; in contrast, rifampin rapidly inactivates free enzyme. Rifampin may thus be used to determine whether an inhibitor of transcription acts by preventing the formation of a preinitiation complex or by interfering with transcription at a subsequent step.

Gal repressor is a specific inhibitor of gal mRNA synthesis. Addition of repressor to λg<sub>gal</sub> DNA prior to CRP and RNA polymerase decreases gal mRNA synthesis to control (minus cAMP) levels (Table I, Lines 1 to 4). This repression is prevented if fucose is added with the repressor; fucose, an inducer of the gal operon, is known to inhibit binding of the gal repressor to λg<sub>gal</sub> DNA (Table I, Line 6). Addition of fucose at the time transcription is initiated (in this experiment, at the time of addition of Mg<sup>2+</sup>) does not restore gal mRNA synthesis (Table I, Line 6).

A preinitiation complex of λg<sub>gal</sub> DNA, CRP, cAMP, and RNA polymerase is resistant to repressor (Table I, Lines 7 to 9). Neither incubation of CRP and cAMP with λg<sub>gal</sub> DNA nor RNA polymerase alone with λg<sub>gal</sub> DNA produces a repressork-resistant complex (Table II). In these experiments, in which a preinitiation complex was allowed to form before repressor was added, the repressor was present for 5 to 7 min before rifampin addition.

The amount of gal mRNA was measured by DNA-RNA hybridization (1). In some experiments the production of gal mRNA was determined by the difference between cAMP-dependent and -independent total RNA synthesis as described previously (11).

**RESULTS**

Incubation of λg<sub>gal</sub> DNA with cyclic AMP, CRP, and RNA polymerase results in the formation of a transcription preinitiation complex at the gal promoter. Transcription of gal DNA can then be initiated by the addition of ribonucleoside triphosphates or Mg<sup>2+</sup> to a complex made in the absence of one of these components. RNA polymerase bound to DNA as a preinitiation complex is relatively resistant to rifampin; in contrast, rifampin rapidly inactivates free enzyme. Rifampin may thus be used to determine whether an inhibitor of transcription acts by preventing the formation of a preinitiation complex or by interfering with transcription at a subsequent step.

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**TABLE I**

**Effect of order of addition of CRP, RNA polymerase, and gal repressor on gal transcription**

<table>
<thead>
<tr>
<th>Time of addition</th>
<th>Gal mRNA</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>CRP +</td>
</tr>
<tr>
<td>7 min</td>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt; +</td>
</tr>
<tr>
<td>14 min</td>
<td>rifampin +</td>
</tr>
</tbody>
</table>

**TABLE II**

**Effect of order of addition of CRP, RNA polymerase, and gal repressor on gal transcription**

Conditions were similar to those of Table I. All of the initial components were preincubated at 0°, the temperature was raised to 37°, and various additions were made at 5-min intervals. Five minutes after the last addition, rifampin and MgCl<sub>2</sub> were added.
addition. Periods of exposure to repressor of as much as 15 min did not produce any significant repression. Since binding of repressor to λgal DNA occurs in less than 5 min under these conditions, the lack of repression cannot be due to insufficient time for repressor-DNA interaction. These results, taken together, suggest that the gal repressor competes with RNA polymerase and CRP at an early step in transcription. The repressor appears to act by preventing the formation of a preinitiation complex; the complex, once formed, is resistant to repression.

The following experiments were performed to rule out alternate interpretations.

1. In the above experiments, a preinitiation complex was formed by preincubating λgal DNA, RNA polymerase, CRP, cAMP, and the 4 ribonucleoside triphosphates in the presence of EDTA. RNA synthesis was initiated by the addition of MgCl₂ in excess of the EDTA concentration, together with rifampin. We assume that the EDTA in the preinitiation mixture, by chelating Mg²⁺, prevents all polyribonucleotide synthesis. To rule out the possibility that EDTA-resistant RNA synthesis was responsible for the refractivity of the preinitiation complex to repressor, we performed similar experiments in which RNA synthesis was initiated by the addition of Mg²⁺ and the 4 ribonucleoside triphosphates. Identical results were obtained; repressor did not inhibit gal mRNA synthesis by a preinitiation complex (data not shown).

2. When fucose is added before the formation of a preinitiation complex, it abolishes the action of repressor. When the order of addition is repressor, then RNA polymerase plus CRP, then fucose together with rifampin plus Mg²⁺, fucose does not relieve repression (Table I, Lines 5 and 6). We interpret these results as indicating that repressor prevents the formation of the preinitiation complex. This interpretation rests on two assumptions: (a) that fucose could remove repressor from λgal DNA in a mixture containing RNA polymerase and CRP; and (b) that this removal is faster than RNA polymerase bound in a preinitiation complex is inactivated by rifampin. (Rifampin inactivates RNA polymerase bound to the gal promoter with a half-life of 25 s (data not shown).) We present data below supporting these assumptions.

The half-life of the gal repressor-DNA complex in the presence of 10 mM fucose is too fast to be measured by existing techniques; our binding experiments show that fucose completely dissociated the complex in less than 25 s (data not shown). To demonstrate that fucose also rapidly dissociated DNA-bound repressor under our transcription incubation conditions, we performed the experiments shown in Fig. 1. In these experiments, repressor was incubated with λgal DNA, then RNA polymerase and CRP were added, and the mixture was incubated for an additional 7 min. Fucose was then added and, at various times, portions were removed and mixed with Mg²⁺ and rifampin; the amount of gal mRNA synthesized was then determined. Fig. 1 shows that gal transcription rises to unrepressed levels in less than 2 min after the addition of fucose, indicating that repressor binding is reversible under our experimental conditions. The rate-limiting step in the derepression by fucose could be either the removal of repressor or the formation of a preinitiation complex. In Fig. 2, we show that the rate of formation of the preinitiation complex is about the same as the rate of derepression by fucose, suggesting that preinitiation complex formation is probably rate-limiting. For simplicity in the experiments shown in Fig. 2, we measured the amount of total RNA made in the presence and absence of cAMP; this difference corresponds to RNA initiated at the gal promoter (as determined by hybridization; data not shown).

The above results are consistent with our hypothesis that repressor prevents preinitiation complex formation. Nevertheless, the fact that our interpretation rests upon a rate difference between two very fast reactions, dissociation of DNA-
bound repressor by fucose and inactivation of complexed RNA polymerase by rifampin, creates sufficient ambiguity to justify another experimental approach. We therefore turned to another inhibitor of RNA polymerase, poly(rI). Like rifampin, poly(rI) binds tightly to free RNA polymerase, preventing it from transcribing DNA (14, 15). Poly(rI), however, is completely without effect on RNA polymerase bound in a preinitiation complex. This is shown in Table III. Note: the addition of poly(rI) prior to the formation of a preinitiation complex completely blocks transcription (Table III, Lines 3 and 4). The addition of poly(rI) after RNA polymerase but before CRP prevents the formation of a complex at the gal promoter and specifically inhibits cyclic AMP-stimulated gal transcription (Table III, Lines 7 and 8). A preinitiation complex at the gal promoter is stable to poly(rI) for at least 7 min (Table III, Lines 5 and 6), whereas all preinitiation complexes are sensitive to incubation with rifampin for the same time period (Table III, Lines 9 and 10).

Unexpectedly, poly(rI) was found to stimulate over-all RNA synthesis. As shown in Table IV, this increase is manifested after a long (20 min) incubation but not after a short (2 min) incubation. Furthermore, the increase is almost entirely due to non-gal RNA; after 20 min, poly(rI) increased total RNA synthesis by 2-fold and gal mRNA synthesis by only 20%. Although the mechanism of poly(rI) stimulation is unclear, we believe that it is likely to act by increasing RNA chain length.

In Table V, we present the results of experiments in which poly(rI) was used to probe the mechanism of action of gal repressor. The addition of repressor to Apga DNA prior to the addition of CRP and RNA polymerase produced a 65% inhibition of gal mRNA synthesis (Table V, Line 2), a level equivalent to that seen in the absence of cAMP (Table V, Line 1). Repression of gal mRNA synthesis is relieved by incubation with fucose for 5 min prior to addition of MgCl₂ and rifampin (Table V, Lines 2 and 3). The derepression by fucose is completely abolished by poly(rI) (Table V, Lines 3 to 5). This result indicates that, as concluded above, gal repressor prevents the formation of a preinitiation complex at the gal promoter.

In most experiments reported in this paper, we observed that the amount of gal RNA made in the absence of cyclic AMP was much higher than that observed by Nissley et al. (4), and that the synthesis of this RNA was not subject to repressor action. We initially considered the possibility that the high
basal level of gal RNA synthesis was due to the higher levels of RNA polymerase used in this study, but this did not turn out to be the explanation. Instead, we found that the cyclic AMP-independent gal RNA synthesis was due to the high concentration (5%, v/v) of glycerol present in our assays. Glycerol is present in the repressor preparation, to which it is added to improve stability. The effect of glycerol on transcription is under study.

**DISCUSSION**

In this paper we have presented evidence, based on in vitro transcription studies, which indicates that gal repressor and RNA polymerase plus CRP compete for binding to the gal operator-promoter region. Incubation of λpgal DNA with gal repressor prior to the addition of CRP, cAMP, and RNA polymerase prevents the formation of a rifampin- or poly(rI)-resistant preinitiation complex and the subsequent transcription of gal DNA. On the other hand, transcription from a preformed preinitiation complex is not inhibited by the subsequent addition of repressor. There is no competition between CRP or RNA polymerase alone and repressor. The resistance of the preinitiation complex to repressor action is not due to formation of oligonucleotides by RNA polymerase; repressor-resistant complexes form in the absence of ribonucleoside triphosphates.

In this discussion, RNA polymerase “binding” to DNA is used synonymously with formation of a preinitiation complex, i.e. a complex resistant to poly(rI) and relatively resistant to rifampin. It is clear that RNA polymerase may interact with DNA in several steps prior to preinitiation complex formation. Which of these stages may be sensitive to repressor is not known.

Experiments on the mechanism of action of the lac, λ, bio, and trp repressors do not present a unified picture. After measuring the amount of β-galactosidase made in vivo with a trp-lac fusion strain, Reznikoff et al. (16) concluded that the lac repressor could interact with the lac operator to block partially lac transcription initiated at a trp promoter. They suggested, therefore, that repressor could occur subsequent to RNA chain initiation. However, similar experiments, in which the expression of the gal, trp (17), or bio (18) operon was initiated at λ promoters, failed to demonstrate an effect of the repressors of these operons on such expression.

Chen et al. (19) directly analyzed the mechanism of action of lac repressor in an in vitro lac mRNA-synthesizing system. They found that, for the wild type lac operon, lac repressor does not compete with RNA polymerase for binding, an observation consistent with the conclusion of Reznikoff et al. (16). With a mutant lac promoter (lac p), however, partial competition between RNA polymerase and lac repressor for binding could be demonstrated (19). Chen et al. (19) suggested that the promoter and operator binding sites are distinct in the wild type lac operon, but overlap in the lac promoter mutant.

Similar in vitro experiments with λ repressor suggested that RNA polymerase cannot bind to λ early promoters when λ repressor is prebound to the λ operators (20, 21). Wu et al. (20) also reported that λ repressor can inhibit transcription from a preinitiation complex. This latter finding is in apparent contradiction to the report by Steinberg and Ptashne (21) that such a complex is resistant to repressor. The discrepancy might reflect the fact that the two groups used genetically different λ repressors.

Our biochemical data suggest that the gal operator and pro-

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5 G. Buttin, personal communication; also S. Adhya, unpublished data.

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**Fig. 3.** Model of gal repressor interaction with RNA polymerase and CRP. Operator is represented by **solid bars** and promoter by **striped bars**. E is the structural gene of epimerase. **Rep** and **RNP** are repressor and RNA polymerase, respectively.
clear that the different repressors, λ, lac, and gal, appear to differ in detail in their mechanisms of action.

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