Cyclic adenosine 3':5'-monophosphate and a specific protein, the cyclic AMP receptor, stimulate the synthesis of a number of inducible proteins in Escherichia coli, including those of the lac and gal operons (1). CRP, in the presence of cyclic AMP, binds to DNA. When RNA polymerase is included in the incubation mixture, a transcription preinitiation complex is formed which is relatively resistant to the action of the antibiotic rifampicin; addition of ribonucleoside triphosphates and Mg²⁺ results in the transcription of lae or gal mRNA from luc or gal DNA templates (3-4). These results suggest that the CRP-cyclic AMP complex promotes mRNA synthesis by binding at the promoter locus of an operon and facilitating the association between RNA polymerase and DNA.

1 The abbreviations used are: cyclic AMP or cAMP, cyclic adenosine 3':5'-monophosphate; CRP, cyclic AMP receptor; lac, the lactose operon; gal, the galactose operon; cyclic GMP or cGMP, cyclic guanosine 3':5'-monophosphate; RNP, RNA polymerase.

SUMMARY

To elucidate further how cyclic adenosine 3':5'-monophosphate (cyclic AMP) and the cyclic AMP receptor are able to stimulate gal mRNA synthesis we have utilized an antiserum specific for cyclic AMP receptor and also cyclic GMP as inhibitors of cyclic AMP and cyclic AMP receptor action. When cyclic AMP, cyclic AMP receptor, DNA, and RNA polymerase are incubated together a rifampicin-resistant preinitiation complex is formed and, upon the addition of Mg²⁺, gal mRNA synthesis occurs. If the preinitiation complex is formed in the absence of nucleoside triphosphates the antiserum is able to dissociate the complex. If the complex is formed in the presence of nucleoside triphosphates (no Mg²⁺), it is converted to a more stable form which is not reversed by the addition of CRP. Cyclic GMP can prevent the formation of the preinitiation complex. However, we find that once the preinitiation complex has been formed cyclic GMP does not inhibit cyclic AMP receptor-cyclic AMP-dependent transcription, suggesting that RNA polymerase stabilizes cyclic AMP receptor-cyclic AMP binding to the gal promoter region.

To understand more fully the early steps in transcription of CRP-cyclic AMP-dependent operons, we performed studies utilizing CRP antiserum to inactivate CRP. Cyclic GMP, which competes with cyclic AMP for binding to CRP (5), was also used as an inhibitor. Our evidence indicates that (a) RNA polymerase stabilizes CRP-cyclic AMP binding to the gal promoter region, and (b) the addition of nucleoside triphosphates to a preinitiation complex converts the complex to a more stable form even when polymerization is prevented by the omission of Mg²⁺.

MATERIALS AND METHODS

Materials—Rifampicin was purchased from Ciba and cyclic GMP was from Boehringer Mannheim. All other chemicals were obtained from sources indicated elsewhere (5). CRP was purified to apparent homogeneity by the procedure of Anderson et al. (5) and has a specific activity of 4400 pmol of cyclic AMP bound per mg of protein. E. coli RNA polymerase (specific activity 350 units per mg) was obtained from Miles Laboratories. λppa25 DNA was extracted from bacteriophage λppa25cI857Sam7 (6) as described by Nissley et al. (3).

Preparation of CRP Antiserum—Antibody was prepared by immunizing rabbits with purified CRP as described elsewhere (6). As might be expected the antibodies obtained from each of four rabbits exhibited different affinities for CRP and differed in their ability to inhibit CRP-cyclic AMP-dependent transcription. Other antibody preparations required incubation periods of up to 30 min to inhibit CRP activity completely.

In Vitro Transcription of Gal Operon—Nissley et al. (3) noted that in a transcription system with λppa25 DNA as template and E. coli RNA polymerase, the addition of CRP and cyclic AMP produced a 15-fold increase in gal mRNA synthesis and a 2-fold increase in total trichloroacetic acid-precipitable [³H]RNA. This increase in total RNA synthesis is due to transcription beginning at the gal promoter and extending into adjacent λ genes. Stimulation of total RNA synthesis therefore provides a quick, reliable estimate of CRP transcription-stimulating activity (3, 5). The assay procedure used is described elsewhere (7). Standard reaction mixtures (0.1 ml) contained 20 mM Tris-HCl, pH 7.9, 15 mM MgCl₂, 100 mM KCl, 0.1 mM sodium EDTA, 0.1 mM dithiothreitol, 0.15 mM ATP, UTP, and GTP, and 0.07 mM CTP, 10,000,000 cpm of [³H]CTP (specific activity 17.9 Ci per mmole), 30 μg of bovine serum albumin, 13 μg of λppa25 DNA, 8.4 μg of E. coli B RNA polymerase, and 4.15 μg of CRP, in either the presence or absence of 10⁻⁴ or 10⁻⁵ M cyclic AMP.

A 0 to 30% (NH₄)₂SO₄ fraction of CRP antiserum (resuspended and dialyzed with 10 mM potassium phosphate buffer, pH 7.8) was
used in studies of the inhibition of CRP-cyclic AMP-dependent transcription. Unfractionated CRP antiserum, as well as normal rabbit serum, contained other components which inhibited overall RNA synthesis. The standard reaction was initiated by RNA polymerase and terminated after a 10-min incubation at 30°C. In experiments involving formation of a preinitiation complex, reaction components were incubated at 30°C for the indicated times and transcription then initiated by the addition of rifampicin (10 μg per ml) together with missing components (nucleotides or Mg++) and further incubated for 10 min at 30°C.

RESULTS

Effect of CRP Antiserum on in Vitro Transcription of Gal Operon—The in vitro synthesis of gal mRNA requires not only gal DNA template (isolated from a λpgal transducing phage) and RNA polymerase, but cyclic AMP and the cyclic AMP receptor protein as well. This synthesis can be detected as an increase in total RNA synthesized from a λpgal DNA template with the addition of cyclic AMP and CRP (see "Materials and Methods"). Fig. 1 shows that the addition of cyclic AMP to reaction mixtures containing CRP, RNA polymerase, and λpgal DNA template produces about a 2-fold increase in total transcription. The addition of increasing amounts of CRP antiserum 10 min prior to the addition of RNA polymerase progressively inactivates CRP as is indicated by the progressive loss in cyclic AMP-dependent transcription. CRP-independent transcription is unaffected by the antiserum. A similar (NH₄)₂SO₄ fraction of nonimmunized rabbit serum has no effect on either CRP-dependent or CRP-independent transcription (data not shown).

Inhibition by CRP Antiserum Depends upon Time of Addition—Inubation of CRP with antiserum for 5 min prior to initiation of transcription with RNA polymerase results in essentially complete inhibition (Table I). If antibody and RNA polymerase are added together, only a 56% decrease results, and if antiserum is added 5 min after polymerization has begun, only a slight inhibition of CRP-dependent transcription is noted. To determine at which reaction step the sensitivity to CRP antiserum is lost, we preincubated CRP with the various components of the transcription mixture prior to the addition of antiserum. Preincubation of CRP with cyclic AMP and λpgal DNA does not protect it from inactivation by antiserum (Table II). Similarly, CRP preincubated 5 min with cyclic AMP and RNA polymerase is also inhibited by antiserum. Thus, any possible association of cyclic AMP-CRP with RNA polymerase in the absence of DNA, or the binding of cyclic AMP-CRP to DNA in the absence of RNA polymerase, does not protect CRP from inactivation by antiserum.

| Time of addition | Inhibition |
|-----------------|-----------|
| -5 min          | 0 min     | +5 min |
| Antiserum A     | RNP       | RNP + antiserum A |
|                 | RNP       | Antiserum A  |

TABLE I

Effect of incubation of CRP with RNA polymerase or λpgal DNA on inhibition of CRP-cAMP-stimulated transcription by CRP antiserum

The transcription system was as outlined under "Materials and Methods." Each reaction mixture contained all of the required nucleotides and the basic salt solution mixed together at 0°C and then brought to 30°C. At zero time CRP, cyclic AMP, RNA polymerase, or λpgalDNA were added as indicated to this reaction mixture followed 5 min later by the addition of a 75-μl sample of a 0 to 30% (NH₄)₂SO₄ fraction of CRP antiserum A where indicated; 75 μl of 10 mM potassium phosphate buffer, pH 7.9, were added in control experiments.

Five minutes after the addition of antiserum either DNA or RNA polymerase was added to initiate the reaction and the samples incubated another 10 min before the reaction was terminated.

| Time of addition | Trichloracetic acid-precipitable |
|-----------------|---------------------------------|
| 0 min           | DNA 99                          |
| 5 min           | DNA 120                         |
| 10 min          | DNA 110                         |

TABLE II

Fig. 1. Inhibition of CRP-cAMP-dependent in vitro transcription by CRP antiserum. Total in vitro transcription with λpgal25 DNA as template was determined as described under "Materials and Methods" in either the presence (●—●) or absence (▲—▲) of 10⁻⁴ M cAMP. The indicated amount of 0 to 30% (NH₄)₂SO₄ fraction of CRP antiserum A (dialyzed versus 10 mM potassium phosphate buffer, pH 7.9) plus 10 mM potassium phosphate buffer, pH 7.9, was added to give a final reaction volume of 0.3 ml. The CRP antiserum A fraction was added 10 min prior to the initiation of transcription by the addition of RNA polymerase. Activity is reported as total counts per min of trichloracetic acid-precipitable [³²P]RNA product.
Effect of CRP Antibody on Stability of Rifampicin-resistant Preinitiation Complex—In these studies we have employed rifampicin, an antibiotic which rapidly inactivates free RNA polymerase but only slowly inactivates RNA polymerase bound to promoter loci on DNA. When DNA containing a lac or gal promoter, CRP, cyclic AMP, and RNA polymerase are incubated together, a complex is formed which is resistant to inhibition by rifampicin. Upon the addition of the missing re-action mixture component (either ribonucleoside triphosphates or magnesium), along with rifampicin, the synthesis of lac or gal mRNA occurs (2-4).

The preinitiation complex formed by λprom DNA, RNA polymerase, CRP and cyclic AMP, which is resistant to rifampicin, is sensitive to CRP antiserum (Table III). The nearly 2-fold increase in RNA synthesis produced by CRP (Table III, Lines 1 and 2) is abolished by antiserum added 5 min after formation of the preinitiation complex (Table III, Lines 3 and 4). This indicates that the presence of active CRP is required to maintain the rifampicin-resistant complex under these conditions.

We then formed a preinitiation complex in the presence of ribonucleoside triphosphates, preventing polymerization by adding sufficient EDTA to chelate all of the Mg\(^{2+}\) present. This complex, unlike the preinitiation complex formed in the absence of the four ribonucleoside triphosphates, was resistant to inactivation by antiserum (Table IV, Lines 1 to 4). The purine ribonucleoside triphosphates alone do not protect; a complex formed with only ATP or ATP + GTP was still unable to synthesize any CRP-cyclic AMP-dependent RNA (Table IV, Lines 5 to 7). These results suggest the preinitiation complex reacts with the four ribonucleoside triphosphates to form a transcription intermediate in which the CRP is either protected from antiserum or is no longer required for synthesis.

Effect of Cyclic GMP on Stability of Rifampicin-resistant Preinitiation Complex—Cyclic GMP, an analogue of cyclic AMP, competes with cyclic AMP for binding to CRP and strongly inhibits transcription from cyclic AMP-CRP-dependent promoters. However, once the CRP-cyclic AMP-λprom DNA-RNA polymerase complex has been formed, the addition of 10^-3 M cyclic GMP has little effect on the stability of this complex whether or not the incubation mixture contained the four ribonucleoside triphosphates (Table V). Nisley et al. (3) have previously reported that a preinitiation complex formed at 17° in the presence of 50 mM KCl and 10 mM MgCl\(_2\) was dissociated by cyclic GMP. Accordingly we assayed the effect of cyclic GMP on CRP-dependent transcription under those conditions. We find again that cyclic GMP is an effective inhibitor only when added prior to the formation of the preinitiation complex (Table VI). This discrepancy is at present unexplained.

| Effect of Cyclic GMP on Basal RNA Synthesis—When 10^-3 M cyclic GMP is added prior to RNA polymerase, cyclic GMP inhibits basal transcription by about 30% (Table VI, Lines 1, 5, and 6). If 10^-4 M cyclic GMP is added after RNA polymerase has been allowed to associate with DNA, no inhibition is observed (Table VI, Lines 1 and 3). When added prior to RNA polymerase 1 mM cyclic GMP not only inhibits basal transcription but also inhibits cyclic AMP-CRP-stimulated transcription (Table VII, Lines 1 to 4). The presence of 1.5 mM GTP inhibits transcription (Table VII, Lines 5 to 7).

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**Table III**

| Time of addition | 0 min | 5 min | 10 min |
|------------------|-------|-------|--------|
| Rif + Mg\(^{2+}\) | 16 | 22 | 18 |
| Rif + Mg\(^{2+}\) + XTPs | 24 | 22 | 18 |
| Rif + Mg\(^{2+}\) + cAMP | 26 | 22 | 18 |
| Rif + Mg\(^{2+}\) + GTP | 26 | 22 | 18 |
| Rif + Mg\(^{2+}\) + XTPs + cAMP | 26 | 22 | 18 |
| Rif + Mg\(^{2+}\) + GTP + cAMP | 26 | 22 | 18 |

* Rif, rifampicin.

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**Table IV**

| Time of addition | 0 min | 5 min | 10 min |
|------------------|-------|-------|--------|
| Rif + Mg\(^{2+}\) | 16 | 22 | 18 |
| Rif + Mg\(^{2+}\) + XTPs | 24 | 22 | 18 |
| Rif + Mg\(^{2+}\) + cAMP | 26 | 22 | 18 |
| Rif + Mg\(^{2+}\) + GTP | 26 | 22 | 18 |
| Rif + Mg\(^{2+}\) + XTPs + cAMP | 26 | 22 | 18 |
| Rif + Mg\(^{2+}\) + GTP + cAMP | 26 | 22 | 18 |

---

**Table V**

| Time of addition | 0 min | 5 min | 10 min |
|------------------|-------|-------|--------|
| Rif, Mg\(^{2+}\) | 16 | 22 | 18 |
| Rif, Mg\(^{2+}\) + XTPs | 24 | 22 | 18 |
| Rif, Mg\(^{2+}\) + cAMP | 26 | 22 | 18 |
| Rif, Mg\(^{2+}\) + GTP | 26 | 22 | 18 |
| Rif, Mg\(^{2+}\) + XTPs + cAMP | 26 | 22 | 18 |
| Rif, Mg\(^{2+}\) + GTP + cAMP | 26 | 22 | 18 |
in the reaction mixture, however, abolished this reduction in basal activity by cyclic GMP, even though the stimulation by cyclic AMP and CRP was completely inhibited (Table VII, Lines 1, 5, and 6).

E. coli RNA polymerase has a Mg\(^{2+}\)-independent binding site for purine nucleoside triphosphates, with a dissociation constant of about 0.15 mM (8, 9). It has been proposed that the 5'-terminal nucleoside triphosphate might bind at this site as a first step in the initiation of RNA synthesis. The inhibition of basal transcription by high concentrations of cyclic GMP may reflect competition for binding with the purine nucleotides at this site. This interpretation is consistent with our observations that (a) DNA-bound RNA polymerase is resistant to cyclic GMP and (b) the effect of cyclic GMP on basal transcription is reversed by GTP.

**DISCUSSION**

We have presented results concerning the mechanism of action of the transcription-positive control factor CRP. Two inhibitors of CRP activity have been used in this study: (a) cyclic GMP, a competitive inhibitor of cyclic AMP binding to CRP, and (b) a specific antiserum to CRP. Previous experiments with in vitro transcription systems have shown that CRP and cyclic AMP stimulate lac and gal mRNA synthesis by promoting the formation of a rifampicin-resistant preinitiation complex between RNA polymerase and DNA (2, 3). CRP has been shown to bind to DNA in the presence of cyclic AMP although specificity for DNA containing cyclic AMP-dependent promoters has not been demonstrated (10, 11). No interaction between CRP-cyclic AMP and RNA polymerase has been detected (11). Thus, our working hypothesis has been that the CRP-cyclic AMP complex binds to DNA facilitating the formation of a preinitiation complex between RNA polymerase and an adjacent site.

The results of the experiments presented here give additional information on the formation and stability of the preinitiation complex.

\[
e^{-} + CRP + DNA \rightleftharpoons CRP-DNA
\]

We have shown that CRP antiserum and cyclic GMP are both able to dissociate completely the cyclic AMP-CRP-DNA complex formed as the product of Reaction 2. Only CRP antiserum is able to inactivate the preinitiation complex (cyclic AMP-CRP-DNA-RNA polymerase) produced by Reaction 3. The ability of cyclic GMP to reverse the cyclic AMP-CRP-DNA complex suggests that the binding of cyclic-AMP-CRP to DNA is relatively weak and is probably undergoing rapid association and dissociation. The dissociated CRP is susceptible to cyclic GMP. Alternatively, cyclic GMP may react with the DNA-bound CRP, perhaps at a different site from the cyclic AMP-binding site (7), causing the dissolution of the complex.

When a preinitiation complex of ApGar25 DNA, RNA polymerase, CRP, and cyclic AMP is formed in the absence of nucleoside triphosphates, the complex is resistant to inactivation by cyclic GMP. This indicates that the presence of RNA polymerase increases the association of CRP-cyclic AMP with DNA. We suggest that the presence of polymerase is required to attain specific binding of CRP-cyclic AMP to the gal promoter region. We interpret the formation of a rifampicin-resistant complex of RNA polymerase at the gal promoter dependent on the presence of cyclic AMP and CRP to be due to an increase in the affinity of RNA polymerase for the promoter. That this interpretation is correct is supported by experiments of Nakanishi et al. (12) in which they have shown that in the presence of cyclic AMP and CRP, RNA polymerase bound to the gal promoter cannot be removed by excess poly(rI), a potent inhibitor of unbound RNA polymerase.

If cyclic AMP and CRP are no longer required once RNA polymerase is specifically bound at the gal promoter, the inability of cyclic GMP to inhibit would also be explained. However, the ability of the antiserum to inactivate the preinitiation complex indicates that CRP is still required at that stage. Once CRP is inactivated, polymerase binding at the promoter becomes unstable and is reversed. The antibody could be acting by (a) recognizing CRP bound to DNA and removing it or inactivating it in situ or (b) by serving as a sink to bind up all
of the free CRP. We favor the former mechanism for if the antibody were simply binding all of the free CRP we would have expected cyclic GMP also to inactivate the preinitiation complex.

The evidence for Reaction 4 is the observation that incubation with the four ribonucleoside triphosphates converts the preinitiation complex to an antiserum-resistant form. In these studies precaution was taken to prevent any phosphodiester bond formation by adding sufficient EDTA to chelate any magnesium which might be in the preliminary reaction mixture. The presence of ATP and GTP, nucleoside triphosphates ordinarily believed to be at the 5' end of mRNA chains, was not sufficient to stabilize this complex to attack by CRP antiserum. Stabilization was observed when all four nucleoside triphosphates were present.

Caution must be exercised in the interpretation of these results, however, for the possibility exists that the stabilization of the complex to attack by CRP antiserum might be due to the synthesis of short oligonucleotides formed in the presence of the four nucleoside triphosphates. To rule out this possibility we have attempted to isolate any small oligonucleotides which might be formed during incubations in the absence of Mg++ (EDTA present). Conditions were identical with those described in Table IV except that [α-32P]ATP was used as labeled substrate. The reaction was terminated by boiling, [α-32P]ATP completely hydrolyzed by ribonuclease-free alkaline phosphatase, and the reaction mixture passed over a Bio-Gel P-2 column to separate [32P]orthophosphate from fractions which would contain nucleotides of greater than a molecular weight 500. The possible oligonucleotide fractions were pooled, lyophilized, and chromatographed on polyethyleneimine-cellulose thin layer plates. No evidence could be obtained to indicate the presence of any oligonucleotide fragments formed in the absence of Mg++.

Additional information also exists which lends support to the view that stabilization is not due to the formation of RNA of a few residues per bound RNA polymerase. Nakanishi et al. (12) found that the preinitiation complex formed in the presence of the four required ribonucleoside triphosphates along with EDTA (no Mg++) was inactivated by incubation with rifampicin for 7 min. So and Downey (13) have previously shown that the formation of a single phosphodiester bond is sufficient to protect RNA polymerase from attack by rifampicin.

The interesting studies of Blattner and Dahlberg (14) and Blattner et al. (15) suggest how the preinitiation complex might be converted to an antibody insensitive form by the ribonucleoside triphosphates. Their experiments indicate that the promoter region of the major leftward early message of bacteriophage λ (presumably the site of RNA polymerase binding) and the site at which RNA polymerization begins are separated. During preincubation the polymerase apparently translocates from its binding site to the site of initiation. Of particular interest was the observation that nucleoside triphosphates, but not Mg++, may be required for this translocation process. Once the RNA polymerase-gal DNA complex has been stabilized, the inactivation of CRP by the antiserum would not cause dissociation of polymerase from DNA, explaining the basis of Reaction 4b.

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