Activation of Transcription at Specific Promoters by Glycerol

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SUMMARY

Glycerol added to a transcription system containing λgal DNA and Escherichia coli RNA polymerase holoenzyme stimulates total RNA synthesis and replaces the requirement for cyclic adenosine 3':5'-monophosphate (cAMP) and cAMP receptor (CRP) for promotion of gal RNA synthesis. The stimulatory effect which is proportional to the concentration of glycerol up to 20% (v/v) occurs at the level of preinitiation complex formation. In stimulating gal transcription, glycerol appears to act at or close to the same site as cAMP and CRP because: (a) glycerol has little effect on gal transcription at saturating levels of cAMP and CRP; (b) although glycerol stimulates transcription from λgal DNA bearing revertible gal promoter mutations, transcription from DNA containing a gal promoter deletion is not stimulated; (c) glycerol stimulation of gal promoter mutants is inhibited by cAMP and CRP; and (d) with either glycerol or cAMP and CRP, transcription of the promoter-proximal galE region precedes transcription of the galKT region.

The effect of glycerol on λ early transcription is to stimulate early r-strand RNA synthesis but not early l-strand RNA synthesis. However, if the DNA template contains a defective sex promoter, the decreased levels of early l-strand RNA are restored to normal by glycerol.

Ethylene glycol, dimethylsulfoxide, sucrose, and 1,3-propanediol, all of which lower the Tm of DNA, also stimulate λearly transcription (6, 9). Another protein factor, rha, is probably involved in the termination of both gal and λ transcription (6, 9).

Previously we have reported that the glycerol present in the gal repressor storage buffer increased gal and total RNA synthesis (4). In this paper we report that glycerol will: (a) substitute for cAMP and CRP to stimulate gal transcription; (b) increase λ early r-strand transcription from the + operator but not early l-strand transcription from the +promoter; and (c) activate transcription from defective promoters in gal or λ DNA. Glycerol acts by stimulating preinitiation complex formation at gal and λ promoters, probably by producing local alterations in the structure of the DNA template.

MATERIALS AND METHODS

Chemicals—Cyclic AMP, [5-3H]CTP (16.7 Ci per mmole) were purchased from Schwarz-Mann; UTP, ATP, GTP, and CTP from P-L Biochemicals; polyinosinic acid from Miles Laboratory; rifampin from CIBA Pharmaceutical Company.

Bacteriophage Strains Used for DNA Extraction—DNA was extracted as described by Nissley et al. (2) from the bacteriophage shown in Table I. This DNA was used as a template for RNA synthesis and was separated into l- and r-strands to detect specific message made in vitro by hybridization.

CRP and RNA Polymerase—Preparation of CRP was described previously (3). Escherichia coli RNA polymerase holoenzyme, factor, and core enzyme were purified according to the method of Becs, et al. (13).

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 7.0), 100 mM KCl, 0.1 mM dithiothreitol, 0.15 mM ATP, 0.15 mM GTP, 0.075 mM [3H]CTP (16.7 Ci per mmole), 100 μg per ml of bovine serum albumin, 8.4 μg per ml of DNA, 12 μg per ml of RNA polymerase, and 4 mM EDTA (to chelate all of the Mg2+ present). Six micromoles per ml of CRP and 0.1 mM cAMP were added where indicated. The reaction mixture was incubated at 37°C for 15 minutes. After incubation, the reaction products were extracted and separated by electrophoresis on 5% polyacrylamide-10% acrylamide slabs.
tion mixture was incubated at 37° for 10 min to form a preinitiation complex. Transcription was started by the addition of MgCl₂ (final concentration, 10 mM) and rifampin (10 μg per ml) and the RNA made was measured after a 10-min incubation, unless otherwise noted. The amount of gal RNA was measured by DNA-RNA hybridization (2). To measure the amount of λ early l-strand or λ early r-strand RNA, the [αH]RNA was first hybridized to filters containing λimm² DNA or λimm₄ DNA, respectively. Portions of the supernatant fluids from these prehybridization mixtures were then hybridized to the l-strand or r-strand of λ DNA by liquid-liquid hybridization. Conditions of filter prehybridization and liquid-liquid hybridization for λ RNA were the same as described in the assay of gal RNA (2).

RESULTS

Glycerol Stimulates Transcription—The DNA of λgal bacteriophage contains both λ and E. coli sequences as shown in Fig. 1. When used as a template for transcription, RNA polymerase by itself will transcribe certain phage sequences but will not transcribe the bacterial gal operon. The addition of cAMP and CRP to the transcription system has no detectable effect on transcription initiated at λ promoters, but stimulates the transcription of the gal DNA sequences at least 10-fold (2). Cyclic AMP and CRP act by increasing the formation of rifampin-resistant preinitiation complexes between RNA polymerase and the gal promoter (2).

We observed that the addition of glycerol to a reaction mixture containing λgal DNA and RNA polymerase stimulated total RNA synthesis (Fig. 2). The stimulation was proportional to the glycerol concentration up to 20% (v/v) glycerol,

![Fig. 1. Schematic genetic map of λgal. The bacterial portion of the λgal is shown by the solid lines and the phage portion by the dashed lines. The direction of transcription is indicated by the wavy arrows. The three structural genes of the gal operon are epimerase (E), transferase (T), and kinase (K). The relative order of the gal operator (O) and promoter (P) is not known. The nonhomologous regions between λ, λimm₄, and λimm₂ are shown in the upper part. The extent of the deletions in the gal26 and gal17 is shown by the vertical dashed lines. galP-3 and galP-211 are mutations in gal promoter. 1 and 3 are mutations in sex promoter.](http://www.jbc.org/)
at which point RNA synthesis was increased 3-fold. This assay measures the number of rifampin-resistant RNA polymerase-DNA complexes formed during a 10-min preincubation. These complexes are thought to represent associations between RNA polymerase and DNA promoter regions (14). Glycerol was therefore increasing the affinity between the promoters and RNA polymerase and/or stimulating the formation of new rifampin-resistant associations between RNA polymerase and DNA.  

**Glycerol Acts at or near gal Promoter**—Fig. 2 also shows that glycerol stimulates the synthesis of RNA complementary to the sense strand (l-strand) of gal DNA only in the absence of cAMP and CRP. This finding supports the idea that glycerol affects promoter-RNA polymerase interactions; if glycerol introduced new RNA initiation sites on DNA, an additive effect of cAMP and CRP and glycerol might have been expected.

We next performed several experiments designed to test the hypothesis that glycerol stimulated the binding of RNA polymerase to promoters.

1. If glycerol stimulated the binding of RNA polymerase to the gal promoter in the absence of cAMP and CRP, then DNA from bacteriophage λgal 245 which is deleted for the gal promoter (as well as for a portion of galE), should not serve as a template for gal RNA synthesis. Indeed, this is the case; neither cAMP and CRP nor glycerol will stimulate gal RNA synthesis with this DNA (Fig. 3). Thus glycerol-promoted gal transcription must initiate within the 326 deletion. Note that while gal RNA synthesis from this DNA was not stimulated by glycerol, total RNA synthesis was increased about 3-fold. This finding indicates that glycerol also activates transcription of λ genes. We shall return to this point below.

2. To characterize further the glycerol-dependent site of gal RNA initiation, we compared the rate and sequence of transcription of portions of the gal operon in the presence of glycerol and in the presence of cAMP and CRP. In these experiments, RNA was synthesized from a λgal DNA template and hybridized to the l-strand of λgal DNA or to the r-strand of λgal 411, which is deleted for the gal region. The former hybridization measures total gal RNA (KTs); the latter, the gal RNA derived from the promoter-distal portion of the operon (KT). As is shown in Fig. 4 the chronology of transcription is identical in glycerol and in cAMP and CRP. galE RNA appears without lag and about 1 min before galKT RNA is seen, a value consistent with prior transcription of the galE gene. Since RNA polymerase in vitro at 37°C catalyzes the incorporation of about 1000 nucleotides per min, we estimate that the initiation site for glycerol-dependent gal transcription is located within a few hundred nucleotide pairs of the cAMP- and CRP-dependent initiation site.

3. The binding of RNA polymerase to promoter sites requires holoenzyme, i.e. core plus sigma. We therefore studied the effect of glycerol on gal transcription with RNA polymerase core enzyme and reconstituted RNA polymerase holoenzyme. The results are presented in Table II. Note that the stimulation of gal RNA synthesis by either cAMP and CRP or glycerol depends upon the presence of sigma; this result lends further support to the idea that glycerol acts at promoter sites.

**Glycerol Activates Defective gal Promoters**—Although glycerol is ineffective in stimulating the template activity of DNA bearing a gal promoter deletion mutation, it strongly stimulates gal transcription from DNA with revertible gal promoter mutations (galP-211, galP-3) (see Ref. 15). As seen in Fig. 5, A and B, DNA extracted from λgalP-211 or λgalP-3 will not support the synthesis of gal RNA in the presence of cAMP and CRP, indicating that they are defective in gal transcription. However, both DNAs are excellent templates for gal transcription when glycerol is added to the preincubation mixture. Note that cAMP and CRP partially inhibit the action of glycerol indicating that they bind to the mutant template. This observation is consistent with the notion that cAMP and CRP and glycerol affect the same DNA region, and in the case of the galP-3 and galP-211 templates, their actions are antagonistic.

Glycerol stimulates transcription from these mutant promoter templates by activating the formation of preinitiation complexes. In the experiments reported in Table III, we used the synthetic polynucleotide, poly(rI), to distinguish between free RNA polymerase and RNA polymerase bound in a preinitiation complex. Poly(rI) has one advantage over rifampin; poly(rI) does not inactivate RNA polymerase bound to a promoter, whereas rifampin eventually inactivates even the bound form of the enzyme (4). Both compounds rapidly inactivate free RNA polymerase.

Note that glycerol activates gal transcription from λgalP-211 DNA when added prior to poly(rI) but not after (Table III, Lines 2 and 3). We conclude that glycerol promotes the formation of gal preinitiation complexes on this template and these complexes: (a) are stable for at least 7 min in the presence of poly(rI); and (b) can form in the absence of the four ribonucleoside triphosphates (Line 4). Preincubation of λgalP-211 DNA with RNA polymerase and cAMP and CRP does not yield a poly(rI) resistant complex; addition of cAMP, CRP, and RNA polymerase prior to poly(rI) and glycerol as shown in Line 5 does not result in gal RNA synthesis. Since cAMP and CRP produce only a partial inhibition of the glycerol-stimulated gal transcription from the template (see Fig. 5A), even when added prior to glycerol (data not shown), we conclude that a preinitiation complex will not form between RNA polymerase and the mutant DNA, even in the presence of cAMP and CRP. The P-211 mutation, is, therefore, a mutation affecting the binding of RNA polymerase or cAMP and CRP to gal DNA whose phenotype is overcome in vitro by glycerol. These data are inconsistent with an alternative model in which the P-211 mutation only affects gal RNA propagation.

**Effect of Glycerol on λ Transcription**—The data presented

### Table II

| Enzyme          | Addition       | Total RNA | galRNA |
|-----------------|----------------|-----------|--------|
| core            | CRP            | 19.6      | 0.78   |
|                 | CRP + cAMP     | 18.5      | 0.60   |
|                 | 15% glycerol   | 24.1      | 1.08   |
| sigma + core    | CRP            | 50.2      | 1.72   |
|                 | CRP + cAMP     | 87.3      | 18.21  |
|                 | 15% glycerol   | 111.0     | 6.90   |

Note: Table II shows the requirement of σ factor for gal RNA synthesis. λgal DNA, nucleoside triphosphates, and the other components described under "Materials and Methods" were mixed at 0°C together with the various additions indicated in the first and second columns. The concentration of RNA polymerase core enzyme and σ factor were 25 μg per ml and 25 μg per ml, respectively. After a 10-min preincubation at 37°C, RNA synthesis was initiated by the addition of MgCl2 and the amount of galRNA and total RNA synthesized after a 10-min incubation at 37°C were determined.

1 Richard Musso, manuscript in preparation.
Effect of glycerol on formation of preinitiation complex

 galP-211 DNA was used as template. The initial components were mixed at 0°, the temperature raised to 37°, and various additions made at 7-min intervals. Seven minutes after the last addition, MgCl₂ and rifampin were added. In the experiment of Line 4, four ribonucleoside triphosphates were omitted from the preincubation mixture. RNA synthesis was started by their addition together with MgCl₂ and rifampin. The final concentrations of glycerol and poly(r1) are 10% (v/v) and 300 µg per ml, respectively. RNP is RNA polymerase.

| Time of Addition | 
|------------------|
| 0 min | 7 min | 14 min | 21 min |
| 1. RNP | glycerol | poly(r1) | glycerol | Mg + Rif* |
| 2. RNP | poly(r1) | Mg + Rif | 4.57 |
| 3. RNP | glycerol | Mg + Rif + XTP* |
| 4. RNP | Mg + Rif | 5.06 |
| 5. RNP | poly(r1) | Mg + Rif | 0.93 |

TABLE III

Effect of glycerol on formation of preinitiation complex

above indicates that glycerol activates transcription from λ DNA as well as from gal DNA. We therefore tested the effect of glycerol on λ early RNA synthesis. Our analysis demonstrates that glycerol stimulates λ early l-strand RNA but not λ early l-strand RNA (Fig. 6). In this experiment, a template which contains the wild type promoters for these regions, χ⁺ and sex⁺, respectively, was used. However, when the template carries a defective promoter for λ early l-strand RNA, sex1 (6) or sex3, the diminished synthesis of this transcript is restored to normal by glycerol (Fig. 7). It is of interest that the sex1 defect, which is less severe than the sex3 defect, is restored by a lower concentration of glycerol. As in the case with galP-3 and galP-211, glycerol must be present during the preincubation period (data not shown). These observations support the idea that glycerol can act directly on defective promoters to stimulate transcription. It further suggests that the sex1 and sex3 mutations affect RNA polymerase binding, in agreement with the data of Chadwick et al. (16).

Mechanism of Action of Glycerol on Transcription—The proportional relationship between glycerol concentration and the stimulation of defective promoters, as well as the difference in glycerol concentration required to restore full activity to sex1 and sex3 mutants, suggested that glycerol works through interactions with DNA rather than with RNA polymerase. Since glycerol has been reported to decrease the Tₘ of DNA (1T), we measured the effect of preincubation temperature on preinitiation complex formation. As shown in Fig. 8, the formation of preinitiation complexes between RNA polymerase and the galP-211 promoter is strongly temperature-dependent; the lower the preincubation temperature, the higher the glycerol concentration required. Prolonging the preincubation time did not increase preinitiation complex formation; evidently the reaction is complete by 10 min.

We also tested the effect of DNA denaturing reagents related to glycerol (17, 18) on both total RNA synthesis and on their ability to “suppress” the effect of the galP-211 mutation. We define suppression here as the restoration of promoter activity to the DNA template. Dimethyl sulfoxide and sucrose are even more potent than glycerol in stimulating total and gal RNA synthesis (Table IV) whereas isopropyl alcohol and n-propyl alcohol inhibited the reaction (data not shown).

Although glycerol and the other reagents mentioned above do reduce the dielectric constant of the reaction medium, this cannot explain their mechanism of action. As shown in Fig. 9, omitting KCl entirely from the mixture does not restore activity to the galP-211 promoter. Nevertheless KCl, which stabilizes the DNA helix (19) and glycerol which destabilizes it, also have antagonistic effects on transcription from the galP-211 template. Stimulation by 15% (v/v) glycerol is maximal when no KCl is added. The inhibition of glycerol promoted gal RNA synthesis by KCl can be overcome by raising the preincubation temperature (Fig. 10).

The relationship between preincubation temperature and λ early l-strand RNA synthesis is demonstrated in Fig. 11A. Transcription from the wild type promoter is independent of temperature over a 20° range, whereas the suppression of the sex3 mutation by glycerol shows a marked temperature de-
Fig. 6 (left). Effect of glycerol on transcription of λ early genes with λsex+λDNA. Conditions were similar to those of Fig. 2 except that λgalP-211, which contains normal sex+ and sex− promoters was used as template and the amount of λ early l-strand RNA (●), λ early r-strand RNA (□), and total RNA (○) was determined as described under "Materials and Methods."  

Fig. 7 (center). Effect of glycerol on transcription from the mutant λsex promoters. Conditions were similar to those of Fig. 6, except that λsex1 DNA (●) and λsex2 DNA (□) were used as template and the amount of λ early l-strand RNA was measured. The results for sex+ (○) were taken from Fig. 6.

### Table IV

Comparison of effect of various solvents on RNA synthesis

A preincubation mixture containing λgalP-211 and RNA polymerase was mixed at 0° together with the various solvents indicated in the first column. After a 10-min preincubation at 37°, MgCl₂ and rifampin were added and total RNA and gal RNA synthesized after a 10-min incubation at 37° were measured. 2.5 M glycerol corresponds to 18.5° (v/v).  

| Solvent               | Total RNA (pmoles CMP) | gal RNA (pmoles CMP) |
|-----------------------|------------------------|----------------------|
| None                  | 25.5                   | 1.06                 |
| 2.5 M Glycerol        | 63.4                   | 8.18                 |
| 2.5 M Ethylene glycol | 60.2                   | 6.54                 |
| 2.5 M Dimethylsulfoxide | 145.2                 | 20.55                |
| 2.5 M 1,3-Propanediol | 58.7                   | 7.53                 |
| 0.37 M Sucrose        | 50.4                   | 5.97                 |

A. C. D. with a midpoint at about 26° and complete suppression at ~ 32°. In the absence of glycerol, no suppression of the sex3 mutation is observed even at 37° (see Fig. 7).

We next compared glycerol suppression of promoter mutations at different preincubation temperatures. The results displayed in Fig. 11B indicate:

1. When the template contains a wild type gal promoter, CAMP and CRP stimulate preinitiation complex formation at a lower temperature than does glycerol.

2. Stimulation of gal RNA synthesis by glycerol with galP+, galP-3, and galP-211 templates shows the same temperature dependence.

FIG. 8 (right). Effect of preincubation temperature on formation of preinitiation complexes at various concentrations of glycerol. A preincubation mixture containing λgalP-211 and RNA polymerase as described under "Materials and Methods" was mixed together with various concentrations of glycerol at 0°. After a 10-min preincubation at various temperatures, MgCl₂ and rifampin were added and the reaction was further incubated for 10 min at 37°. gal RNA made with 20% (v/v) (●), 12.5% (v/v) (○), and 5% (v/v) (□) glycerol, respectively.

Fig. 9. Effect of KCl concentration. A preincubation mixture containing λgalP-211 and RNA polymerase and various concentrations of KCl, as indicated on the abscissa, was mixed where indicated with 15% (v/v) glycerol at 0°. After a 10-min preincubation at 37°, MgCl₂ and rifampin were added and the reaction was further incubated for 10 min at 37°. Total RNA made with (●) or without glycerol (■). gal RNA made with (○) or without glycerol (●).
FIG. 10 (left). Effect of KCl on formation of preinitiation complexes in the presence of glycerol. Conditions were similar to those in Fig. 8, except that the concentration of glycerol was fixed at 15% (v/v), and either KCl was omitted (C) or 0.1 M KCl was used (O).

FIG. 11. Effect of preincubation temperature on formation of preinitiation complex. Conditions were similar to those of Fig. 8, except for the following: A, λgalP-211 (O) or λsez3 DNA (C) was used as a template and the concentration of glycerol was 15% (v/v). λ early l-strand RNA was measured as described under “Materials and Methods.” B, a preincubation mixture containing λgal DNA was mixed either with 15% (v/v) glycerol (O) or with cAMP and CRP (C). Similar mixtures containing λgalP-211 DNA (O) or λgalP-3 DNA (C) were mixed with 15% (v/v) glycerol. After a 10-min preincubation at the indicated temperatures, MgCl₂ and rifampin were added and the reaction was further incubated for 10 min at 37°.

3. Suppression by glycerol of the λsez3 mutation is achieved at a lower temperature than the suppression of galP⁻ (compare Fig. 11, A and B). These results indicate that the sez3 mutation decreases the binding of RNA polymerase to the sex promoter while the galP-3 and galP-211 mutations affect the interaction between the gal promoter region and CAMP and CRP. Glycerol would then suppress the galP mutations indirectly by converting the gal promoter region to a CAMP and CRP independent form. An alternate possibility is that glycerol stimulates gal transcription at a second site close to the CAMP and CRP dependent promoter. This model, however, requires a second, ad hoc, assumption that activation of one promoter in gal inhibits the activity of the other.

DISCUSSION

We have reported in this paper that the presence of glycerol in a purified in vitro system increases transcription by stimulating the formation of preinitiation complexes at bacterial and λ promoter regions. We have studied the transcription of a variety of operons and found: (a) the transcription of λ early r-strand RNA is strongly stimulated by glycerol; (b) the λ early l-strand transcription is not stimulated. However, when this transcription is reduced by the defective promoter mutations, sez1 and sez3, activity is restored by normal by glycerol; and (c) for E. coli gal transcription, glycerol can replace CAMP and CRP and will also restore gal RNA synthesis to normal, when defective promoter mutations are present.

Glycerol appears to affect promoter sites rather than to cause nonspecific initiation of transcription because: (a) transcription factor sigma, which is required for the stable binding of RNA polymerase to promoter sites, is also required for the glycerol effect; (b) at saturating concentrations of CAMP and CRP where the gal promoter is fully active, little increase in gal transcription is seen when glycerol is also present; (c) glycerol also fails to stimulate gal transcription from a template deleted for the gal promoter region; (d) transcription of the galR region precedes by about 1 min the transcription of the promoter-distal galKT region with either CAMP and CRP or glycerol; (e) two defective λ promoter mutants, sez1 and sez3, show different glycerol concentration dependence curves.

We believe that glycerol acts by changing the conformation of the DNA template rather than altering the properties of RNA polymerase: (a) the stimulation of RNA synthesis is proportional to the concentration of glycerol and, in the case of sez1 and λsez3, the more defective the mutant the higher the required glycerol concentration; (b) whereas preinitiation complex formation is not markedly temperature-dependent (over a 8-37° range, data not shown), glycerol-stimulated complex formation is quite temperature-dependent; (c) the midpoint of the temperature transition is lowered either by increasing the glycerol or reducing the salt concentration. Glycerol and salt have antagonistic effects on the stability of DNA, the former reducing and the latter elevating the Tm. Other denaturing agents, i.e. sucrose, ethylene glycol, dimethylsulfoxide, and 1,3-propanediol (17, 18), also stimulate both gal and total RNA synthesis.

The effect of ethylene glycol on the Tm of DNA and its apparent conversion of DNA from the B to the C form (20) reflect a general change in the structure of DNA. In light of this, then would glycerol act specifically to activate promoter regions? One possibility is that glycerol produces a general alteration in the structure of DNA which results in the activation of certain promoter regions. Another possibility is that the promoter regions are uniquely affected by glycerol. There is evidence that the promoter loci might be palindromic and therefore have an atypical secondary structure. In the lac promoter-operator
regions, palindromic symmetry has been proposed by Sadler and Smith (21) on the basis of genetic evidence.

In the case of λ, the σ41 and σ23 mutations eliminate a site of cleavage of the Hemophilus influenzae enzyme restriction; restriction nucleases are known to act at sites of rotational symmetry (22).

The apparent activation of the λ x+ promoter by glycerol is of some theoretical interest. If glycerol acts on wild type promoters only to replace positive control factors, then λ early r-strand transcription may normally be controlled by elements analogous to cAMP and CRP. However, transcription of the λ cI gene, thought to be under positive control, is not stimulated by glycerol (data not shown).

It is known from the work of Brody and Leautey (23) that high concentrations of ethylene glycol, which inhibit over-all transcription from T4 DNA templates, stimulate the synthesis of new GTP-initiated RNA molecules and that this stimulation requires σ factor. Whether this may occur with our templates and under our experimental conditions, where glycerol is stimulatory, remains to be determined. Preliminary evidence indicates that 15% (v/v) glycerol stimulates preinitiation complex formation of T2 and calf thymus DNA templates (data not shown).

The marked effect of glycerol on transcription and its ubiquitous use as a stabilizer of proteins or as a component of gradient centrifugation mixes should be noted. The present study was, in fact, initiated by the observation that the glycerol present in gal repressor preparations antagonized the action of the repressor. Finally our observation that glycerol and related substances can influence specifically the transcription of DNA might prove useful in elucidating the mechanism of initiation of RNA synthesis.

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