Enhancement of Transcription Termination Factor Rho Activity with Potassium Glutamate*

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The efficiencies of rho action as a termination factor during transcription in vitro of several DNA templates were determined as a function of the concentration and type of electrolyte ions. The termination efficiencies with λ D R, and the promoter proximal lacZ intragenic terminators were significantly higher with 0.1–0.2 M potassium glutamate than with the optimal concentrations of KCl (0.05 M) or potassium acetate (0.15 M). Similar high efficiencies were obtained with salts of other acidic amino acids but not with a salt of N-acetylglutamic acid or with a mixture of 0.15 M potassium acetate and 0.15 M glycine, and termination was inhibited completely when 0.12 M KCl was present along with 0.12 M potassium glutamate. The salts that give high termination efficiencies have two properties in common; they consist of anions that are zwitterions, and they are weak chelators of Mg2+ ions. The increase in termination efficiency with potassium glutamate can be ascribed mainly to a facilitation of the reactions of rho with RNA that are coupled to ATP hydrolysis, as the rate of ATP hydrolysis with isolated transcripts as cofactors was about five times higher with 0.15 M potassium glutamate than with 0.05 M KCl, whereas the rates of chain elongation, the general stability of the transcription complexes, and the binding affinity of rho with the transcripts were all very similar under the two conditions. Further analysis revealed that the activation of ATP hydrolysis is an outcome of a shift in the optimum magnesium salt concentration from 0.5 mM with 0.05 M KCl to 4 mM with 0.15 M potassium glutamate. Since glutamate is a relatively weak counterion for cationic groups in proteins, potassium glutamate can be used at 0.15 M without inhibiting the binding of rho to RNA. At that concentration, it serves to buffer the level of free Mg2+ available to stabilize RNA secondary structures that are known to impede rho action on RNA. The two special properties of glutamate together create conditions that allow rho to terminate transcription in vitro at an efficiency that matches the in vivo efficiency with use of a physiological level of K+ ions.

Termination of transcription is an important process in the control of gene expression in prokaryotic cells (Adhya and Gottesman, 1978; Platt, 1986; Friedman et al., 1987). In Escherichia coli, the protein factor rho is responsible for terminating RNA synthesis at many though not all termination sites (Roberts, 1969; Rosenberg and Cour, 1979). Rho is an RNA-binding protein (Richardson, 1970, 1990) that mediates dissociation of transcripts from RNA polymerase molecules paused at termination sites (Richardson and Conaway, 1980). This action is very sensitive to the ionic conditions; rho-dependent terminators that are recognized efficiently in solutions containing 0.05 M KCl are substantially less effective in solutions containing 0.15 M KCl and are virtually inactive in solutions containing 0.2 M KCl (Richardson, 1970; Galuppi et al., 1976; Lau et al., 1982). Since the concentration of K+ ions in E. coli is at least as high as 0.2 M (Epstein and Schultz, 1965; Richey et al., 1987), the strong inhibition in vitro with 0.2 M KCl may be due to an effect of the chloride ions. Recently, Leirimo et al. (1987) demonstrated that steps in the transcription initiation process that are known to be decreased by elevated concentrations of KCl are much less sensitive to the corresponding concentrations of potassium glutamate (KGlu). We have thus examined whether rho factor can function effectively with a physiological level of K+ ions if KGlu is used in the reaction mixture instead of KCl. The results indicate that glutamate is a much more suitable anion than chloride for in vitro studies of rho-dependent transcription termination.

EXPERIMENTAL PROCEDURES

Enzymes, DNA, and Biochemical Reagents—E. coli RNA polymerase was purified from E. coli MRE600 as previously described by Andrews and Richardson (1985). E. coli MRE600 rho protein was purified as previously described by Finger and Richardson (1981).

The DNA template containing the lac UV5 promoter and the first 473 bp of the lacZ gene was the 1250-bp BstEII DNA fragment isolated from pMC1 as described previously (Rusheusser and Richardson, 1989). The DNA containing λ Ra, the λ-cro gene, and λR was the 560-bp HindII fragment isolated from pCYC2 (Chen and Richardson, 1987). DNA containing a 70-bp deletion extending into the rut regions of λ-tR1 (ΔAR70 DNA) was the 490-bp HindII fragment isolated from pCYC2ΔAR70 (Chen and Richardson, 1987). DNA containing the intragenic trpE terminator was the 2150-bp EcoRI-HindIII fragment isolated from pNT4043 (Tsuchiya et al., 1989). It consists of the 370-bp HpaII segment of λ-DNA carrying P, joined to a 1733-bp segment of the E. coli trp operon DNA extending from nucleotide 106 of trpE to nucleotide 275 of trpD. T7D111 DNA was prepared as described by Andrews and Richardson (1985). Plasmid pLZ1 was constructed by ligation of a 459-bp EcoRI fragment that contains bp 1 to bp 459 from lacZ to EcoRI and phosphatase-digested pT7/T3–18 (Bethesda Research Laboratories). In this construction, the first base pair of the lacZ sequence is at bp 10 with respect to the start site for transcription with T7 RNA polymerase. The sequence of the first nine nucleotides of the RNA molecules transcribed from the DNA by action of T7 RNA polymerase is GGGAGACCGG... This plasmid was isolated from a transformed clone of DH5αF'.

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1 The abbreviations used are: KGlu, potassium glutamate; bp, base pairs; KAc, potassium acetate.
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(Hanahan, 1983) by the procedure described by Faus and Richardson (1989).

Unlabeled ribonucleoside triphosphates were purchased from Boehringer Mannheim and [a-32P]UTP was from ICN Chemical and Radioisotope Division. Rifampicin was from Ciba-Geigy Corp. Potassium L-glutamate, sodium L-glutamate, potassium L-aspartate, d-glutamic acid, and N-acetyl-L-glutamic acid were purchased from Sigma. All were made up as 1.5 m stock solutions and adjusted (to pH 7.8) with KOH or NaOH, as appropriate, so that a 0.15 m solution had pH 7.8. All other chemicals were standard reagent grade.

In Vitro Transcription.—For reaction mixtures used to study the efficiency of KC1, the other reaction salts consisted of 0.04 M Tris-HCl buffer, pH 8.0, and 4 mM MgCl2. For reaction mixtures used to study the effects of KGl and other electrolytes besides KC1, the other reaction salts were 0.04 M Tris-acetic acid buffer, pH 8.0, and 4 mM magnesium acetate. The reaction mixtures were prepared by mixing DNA with RNA polymerase and rho, where indicated, in 45 M2 of reaction solution containing reaction salts, 0.1 mM diethiothreitol, 0.1% (w/v) acetylated bovine serum albumin and, 0.1% (w/v) Nonidet P-40. After preincubation for 10 min at 37 °C, 5 µl of a mixture of nucleoside triphosphates (adjusted to pH 7 with KOH) with 10 µg/ml of rifampicin was added to give a final concentration of 260 µM each of ATP, GTP, and CTP, and 20 µM of [a-32P]UTP (5 µCi/ nmol). After incubation for 20 min, the reactions were terminated by adding 0.110 ml of 0.4% sodium dodecyl sulfate in 60 mM Na,EDTA and 40 µl of 0.25 mg/ml of E. coli tRNA. RNA was purified and analyzed by gel electrophoresis as described previously by Andrews and Richardson (1988). Termination efficiency, the percent of RNA polymerase encountering a rho terminator and terminating at the site, was determined from integration of microdensitometer scans of a sample track of the autoradiogram. The cumulative efficiency for n terminators was determined with the formula $(1 - f_1; f_2 \cdots f_n)100$ where $f_i$ is the fraction of RNA polymerase molecules encountering the ith terminator that stops at that terminator.

Synthesis of RNA Molecules Used for Binding and ATPase Studies.—Procedures for the synthesis and isolation of 32P-labeled and unlabeled transcripts of pLZl and pIF2 DNAs are presented in Faus and Richardson (1989). LacZ-217 was transcribed from HaclI-digested plZ1; lacZ-176 RNA was from Pstl-digested plZ1; and λ-cro-378 RNA was from TsqI-digested plF2. All transcripts were synthesized by action of T7 RNA polymerase. Rho-RNA binding and rho-ATPase assays were performed as described by Faus and Richardson (1989).

RESULTS

Enhanced Rho Activity with 0.15 M Potassium Glutamate— Two sets of rho-dependent terminators that have been extensively characterized are λ-tRN (Lau et al., 1982, Morgan et al., 1983a, Chi and Richardson, 1987) and the lacZ intragenic terminators (Rutshusher and Richardson, 1989). Under the standard conditions that have been used previously, with 0.05 M KCl, 4 mM MgCl2, and 0.04 M Tris-HCl, pH 8.0, rho is highly efficient at causing termination at λ-tRN, during transcription of a λ-cro gene DNA template in vitro, but is only moderately efficient at the lacZ terminators. With 0.15 M KCl, rho is virtually inactive at either terminator. To determine whether rho can act efficiently with physiological levels of K+ ions when the counterion is glutamate rather than chloride, the yields of λ-cro and lacZ RNA molecules were measured after transcription of λ-cro and E. coli lacZ DNA restriction fragments, respectively, in vitro in mixtures containing 0.15 M potassium glutamate, 0.04 M Tris-acetic acid buffer, pH 8.0, and 4 mM magnesium acetate. The RNA products isolated from reaction mixtures containing different amounts of rho factor were separated by gel electrophoresis. The results (Fig. 1 for λ-cro; Fig. 2 for lacZ) show that with both sets of terminators, rho acts with high efficiency in 0.15 M potassium glutamate. For comparative purposes, the same templates were transcribed in vitro with the same range of rho concentrations under the "standard" conditions (0.05 M KCl, 4 mM MgCl2, and 0.04 M Tris-HCl buffer, pH 8) and under "high KCl" conditions (0.15 M KCl, 4 mM MgCl2, and 0.04 M Tris-HCl buffer, pH 8). The results confirm that rho acts efficiently at λt1 with 0.05 M KCl, that it acts with poor efficiency at the lacZ terminators with 0.05 M KCl, and that it is virtually inactive at all the rho-dependent terminators with 0.15 M KCl. The most striking observation is that rho acts considerably more efficiently at the lacZ terminators with 0.15 M KGl than with 0.05 M KCl (Fig. 2; quantitated in Table I). Although less obvious, rho also acts more efficiently at λtR, with 0.15 M KGl than with 0.05 M KCl. This is evident from comparison of relative yields of terminated transcripts with intermediate levels of rho; in 0.05 M KCl, as much as 180 fmol of rho was needed to reduce readthrough of sites I, II, and III to less than 10% while only 36 fmol of rho was sufficient to achieve the same level of effectiveness with 0.15 M potassium glutamate (Fig. 1). These observations demonstrate that rho can function efficiently with physiological levels of K+ ions when the anion is glutamate and that rho is significantly more effective with 0.15 M KGl than with 0.05 M KCl.

The amounts of rho needed for termination at λtR are low enough for a catalytic, rather than stoichiometric, action of hexameric rho. Although the reaction mixture contained 0.15 pmol of RNA polymerase, activity measurements indicated that it was about 50% active. Thus, the activity of about 75 fmol of RNA polymerase was terminated by action of 36 fmol (as hexamer) of rho.

The lacZ DNA template used also contains a weakly effective rho-independent terminator which yields a transcript with 421 nucleotides (RI 421). The efficiencies of termination at that site in the absence of rho were 57, 37, and 50% in 0.05 M KCl, 0.15 M KCl, and 0.15 M KGl, respectively (from data shown in Fig. 2). These results indicate that this rho-independent terminator is also sensitive to ionic conditions but is about as active in 0.15 M KCl as in 0.05 M KCl.

With similar assays, we have determined that rho also acts efficiently at T7 DNA early gene rho-dependent terminators (Andrews and Richardson, 1985) as well as at trpE gene intragenic terminators (Tsurushita et al., 1989) with 0.15 M KGl (data not shown). Thus, the ability to function in 0.15 M KGl is apparently a general characteristic of rho-dependent terminators.

To determine the concentration of KGl that allows the most efficient utilization of the rho-dependent terminators in lacZ, the BstEI1 DNA fragment containing the lacZ′ transcription unit was transcribed in vitro in several reaction mixtures, each containing a fixed amount of RNA polymerase and rho and different concentrations of KGl from 0 to 0.25 M. An intermediate level of rho was used in order to reveal slight differences in termination activity. The results (Fig. 3) show that the KGl optimum is broad. The yield of the four rho-terminated transcripts varied little in the range from 0.1 to 0.25 M KGl and decreased outside that range. Thus, the optimum concentration of KGl extends to a level that matches the physiological concentration of K+ ions.

The results in Fig. 3 show that rho was very inefficient in the reaction mixture that had no KGl. A similar inefficiency was observed in rho action with no added potassium glutamate, when T7 D111 DNA was the template (data not shown). The major electrophoreses present in those reaction mixtures were magnesium acetate (4 mM) and Tris-acetic acid buffer, pH 8.0 (40 mM). The severe inefficiency of rho under these conditions is not a consequence of a lack of K+ ions because during transcription of T7 DNA rho is nearly as effective in reaction mixtures containing MgCl2 and Tris-HCl buffer without KCl as in reactions with those same electrophores with 0.05 M KCl (Galluppi et al., 1976). This suggests that with...
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**Fig. 1.** Rho factor dependence of termination at λ-tR1 in reaction mixtures with various electrolytes. *32P*-labeled RNAs were prepared by transcription of 0.25 pmol of 560 bp HindII λ-DNA fragment isolated from pCYC2 DNA with 0.15 pmol of RNA polymerase for 20 min at 37 °C in standard reaction mixtures containing the indicated concentrations of KCl or potassium glutamate (KGlu), and indicated amounts of rho factor and were separated by electrophoresis in an 8 M urea, 5% polyacrylamide gel. The symbols RT, I, II and III indicate the position of transcripts terminated at the end of the template (read-through) and at the three stop points of λ-tR1, respectively. The autoradiogram was cropped to save space; no RNAs shorter than the 290-nucleotide transcript terminated at site I were detected.

**Fig. 2.** Rho factor dependence of termination at the promoter proximal lacZ intragenic terminators in reaction mixtures with various electrolytes. *32P*-Labeled RNAs were prepared by transcription of 0.5 pmol of the 1250-bp BstEII lacZ DNA fragment with 0.3 pmol of RNA polymerase for 20 min at 37 °C in standard reaction mixtures containing the indicated concentrations of KCl and potassium glutamate (KGlu), and indicated amounts of rho factor and were separated by gel electrophoresis. The symbols RT, RD, and RI indicate positions of transcripts initiated at Plac and terminated at the end of the template and at various rho-dependent and rho-independent sites, respectively, with numbers indicating the lengths of the specific transcripts.

Termination efficiencies were determined for RNA analyzed in Fig. 2 in the reaction mixtures with the indicated concentrations of KCl or KGlu and with 1 pmol of rho. The sites are indicated by the symbols RD or RI, for rho-dependent and rho-independent, respectively, and by the bp coordinate in the lacZ gene (length of transcript in nucleotides).

| Electrolyte | Termination efficiency (%) at |
|-------------|-------------------------------|
|             | RD180 | RD220 | RD379 | RD463 | RI421 |
| 0.05 M KCl  |  4    |   10  |   42  |   62  |  51   |
| 0.15 M KCl  | <2    | <2    | <2    |  24   |  43   |
| 0.15 M KGlu | 44    |   76  |   81  | (70)* | (60)* |

* These are approximate values because very few RNA polymerase molecules encountered these terminators under these conditions.

**Fig. 3.** Determination of the optimum concentration of potassium glutamate for rho-dependent termination at the lacZ′ intragenic terminators. *32P*-Labeled RNAs, prepared as described in Fig. 2 in reaction mixtures with either 0.2 pmol of rho or no rho and potassium glutamate as indicated, were separated by gel electrophoresis.

* Rho Activity with Other Salts of Acidic Amino Acids—One of the distinctive features of potassium glutamate as a coun-
Termination efficiencies were determined from microdensitometric scans of autoradiograms of sample tracks of \(^{32}\)P-labeled RNA prepared by transcription of the 1250 bp \(E.\) coli \(lacZ\)' DNA fragments for 20 min at 37 °C in standard chloride-free reaction mixtures containing 0.15 M concentrations of the indicated electrolytes and 0.5 pmol of rho and separated by gel electrophoresis. The sites are indicated as described in Table I.

Table II

| Electrolyte                  | Termination efficiency (%) at |
|------------------------------|--------------------------------|
|                              | RD180 | RD220 | RD379 | RD463 | RD421 |
| Potassium-L-glutamate        | 38    | 55    | 55    | (42)^a | (30)^a |
| Potassium-L-aspartate        | 25    | 46    | 70    | (34)^a | (40)^a |
| Sodium-L-glutamate           | 6     | 17    | 38    | 29    | 34    |
| Potassium-D-glutamate        | 3     | 17    | 62    | 11    | 30    |
| Potassium acetate and glycine | <2    | <2    | <2    | 10    | 21    |
| Potassium N-acetyl-L-glutamate | <2    | <2    | <2    | <2    | 10    |

^a These are approximate values because very few RNA polymerase molecules encountered these terminators under these conditions.

Termination is that it contains a zwitterionic group in the same molecule. Since the presence of the zwitterionic group could greatly influence interactions with water, rho action in the presence of 0.15 M concentrations of other similar salts was tested using the intragenic lacZ terminators as the test system. The results, tabulated as termination efficiencies at the various lacZ stop points (Table II), indicate that with 0.15 M potassium L-aspartate, another "natural" cellular component, rho was nearly as active at all the termination points as with 0.15 M potassium L-glutamate. Although rho was less active with sodium L-glutamate and potassium D-glutamate than with potassium L-glutamate, it was still considerably more active with those salts than with potassium acetate or KCl. Thus, potassium glutamate is not unique in its ability to allow rho to function efficiently. These results support the notion that anions that are also zwitterions may have special interactive properties that make them particularly suited for rho function. The possibility that rho function could be activated by a mixture of a zwitterion and the salt of a carboxylic acid was ruled out by the finding that termination activity in a reaction mixture containing 0.15 M glycine and 0.15 M potassium acetate was even lower than in reaction mixtures with 0.15 M potassium acetate alone (Table II). Thus, the zwitterionic group has to be directly attached to the anionic group.

At pH 8, a small fraction of the acidic amino acids exist in the dianionic form (i.e. as glutamate^-2^), which can bind Mg^2+^ ions. We show in a subsequent section that this ability to bind Mg^2+^ ions has a profound effect on the activity of rho-ATPase with RNA and consequently on the termination process. To test whether a chemically similar chelator that cannot exist in a form with a zwitterionic group can also cause efficient rho-dependent termination, yields of lacZ transcripts were measured with reactions containing 0.15 M potassium N-acetylglutamate. The results indicate that rho was completely inactive with this salt (Table II). This suggests that the other ionic properties of glutamate and the acidic amino acids besides their abilities to chelate Mg^2+^ ions are also important for rho function.

Chloride Ions Inhibit Termination with Glutamate Present—To differentiate whether potassium glutamate is serving as a specific activator or is merely a more effective counter ion than chloride, the yields of RNA products from transcription of the lacZ DNA fragment in the presence of rho in reactions containing a mixture of 0.125 M potassium glutamate and 0.125 M KCl were compared with the yields in reaction mixtures containing 0.125 M or 0.25 M potassium glutamate or KCl alone. The results (Fig. 5) show that rho was virtually inactive in 0.125 M KCl or in 0.125 M KCl supplemented with 0.125 M potassium glutamate. Thus, the strong activity that was found with 0.125 M potassium glutamate was completely overcome by the inhibition caused by chloride. Since rho activity was only slightly reduced with 0.25 M KGluc, the lack of activity with the mixture cannot be attributed to the high K^+^ ion concentration. It should be noted that these reactions were performed under conditions of free initiation between RNA polymerase and DNA. Thus, the low yield of all transcripts with the mixture and total lack of transcription with 0.25 M KCl that were found are expected results based on the known sensitivity of transcriptional initiation to the moderate to high levels of KCl concentration (Richardson, 1966). Overall, these experiments suggest that glutamate and chloride are competing in their effects on rho function and that chloride is a more potent competitor than glutamate.

Transcriptional Pausing and Stability of the Transcription Complex with Potassium Glutamate—The efficiency of rho action at particular points on a DNA template is determined in large part by the dynamics of RNA chain elongation and possibly the relative stability of the ternary complex. Since both of these properties of the RNA polymerase reaction could be sensitive to the electrolyte conditions, we measured the rates of RNA chain elongation and tested for instabilities of ternary complexes during transcription of a lacZ' DNA fragment with \(E.\) coli RNA polymerase in solutions with either 0.05 M KCl or 0.15 M KGluc as the major electrolyte. In both solutions, the magnesium salt concentration was 4 mM, a concentration that allows relatively rapid initiation of transcription with \(E.\) coli RNA polymerase. To follow the elongation reaction, the lacZ' DNA fragment was preincubated with RNA polymerase to allow formation of open (rapid start) complexes, and samples were removed at various times after adding nucleoside triphosphates with rifampicin (to block initiation by RNA polymerase molecules not in complexes) for analysis by polyacrylamide gel electrophoresis. The results (Fig. 6) show that the RNA chains follow almost identical...
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Fig. 5. Inhibition of rho-dependent termination at the lacZ' intragenic terminators with KCl in the presence of potassium glutamate. 

Fig. 6. Kinetics of lacZ RNA elongation in vitro in 0.05 M KCl and 0.15 M KGlu. 

Courses of elongation in the two reaction mixtures. The lengths and relative amounts of the predominant intermediates at various times are nearly the same. Those intermediates arise from elongation pauses at 180, 220, 265, 340, 379, 421, and 463 bp downstream from the lacZ start site (Ruteshouser and Richardson, 1989). The rate of clearance through a pause site can be estimated from the slope of the line through a plot of log (RNA) versus time for a particular RNA, in which (RNA) is the relative intensity of an autoradiographic band determined by densitometry. This rate is conveniently expressed as a relaxation time, the negative reciprocal of the slope of the plot (Morgan et al., 1983b). The relaxation times for the pause points at 180, 220, and 379 bp were ~5, ~5, and 5–10 s, respectively, with 0.05 M KCl and essentially the same with 0.15 M KGlu. Thus, the durations of pausing at the first three lacZ rho-dependent termination stop points were not significantly different in the two electrolyte solutions.

To obtain evidence for inherent instabilities of the transcription complexes at the termination stop points in 0.15 M KGlu, we followed a procedure developed by Arndt and Chamberlin (1990), which measures the ability of RNA polymerase to continue elongation after being arrested for a period of time. This arrest was effected by the addition of 20 mM EDTA 1 min after initiation. After further incubation for 1 h, 20 mM magnesium acetate and a mixture of all four nucleoside triphosphates at 0.5 mM each were added to allow continuation of chain growth by undissociated transcription complexes. The results (Fig. 7) show that, in both 0.05 and 0.15 M KGlu, all the transcripts from RNA polymerase that were arrested at points from 180 to 379 bp from the start point were readily elongated further. Although many RNA polymerase molecules continued to the end of the template to yield transcripts with 610 nucleotides, some were terminated at the rho-independent terminator at bp 421 while others were terminated at points between bp 421 and the end of the template, points that are not used under the normal conditions of transcription. Since we were concerned primarily with the function of the rho-dependent sites that cause termination at bp 180, 220, and 379, we did not explore that curious observation further. The purpose of this experiment was to determine whether complexes arrested at any one of those rho-dependent stop sites were unstable enough in 0.15 M KGlu to have half-time of 1 h or less. The results indicated that the complexes at those points all have stabilities with half-lives considerably longer than 1 h. Because of the problems with nuclease contaminants we did not explore longer incubations. Thus, we conclude that transcription complexes paused at the lacZ rho-dependent stop points are not exceptionally unstable in 0.15 M KGlu.

The Affinity of Rho for Partial LacZ Transcripts—The
binding of rho to a nascent RNA is an essential step in the termination process, a step that is known to be particularly sensitive to ionic conditions (Faus and Richardson, 1989). The characteristics of the RNA binding reaction of rho can be measured directly with a membrane filter retention assay. This assay can thus be used to determine whether the differences in the efficiency of rho action are related to the affinities of rho for lacZ RNA under various conditions. We prepared lacZ RNA molecules by transcription in vitro of restriction fragments that contained the T7 RNA polymerase promoter fused to lacZ. One RNA was transcribed from a DNA that ended at the PvuI site at bp 176 in lacZ while another was from a DNA that ended at the HaeII site at bp 217. These RNAs serve as models for the nascent transcripts that are terminated by rho action at bp 180 and 220, respectively. Because of the nature of the fusion, they have nine extra non-lacZ nucleotides at their 5' end. The results (Fig. 8) show that the binding of rho to the lacZ 217 transcript is qualitatively different in the two solutions. With 0.05 M KC1, all the binding points fall close to a curve for a standard unimolecular binding reaction with $K_a = 1.9 \times 10^6$ M$^{-1}$ and a filter retention efficiency of 41%. With 0.15 M KGl, however, only the binding points with the higher concentrations of rho fall near a curve for a standard binding reaction, this one with $K_a = 1.6 \times 10^6$ M$^{-1}$ and a retention efficiency of 52%, whereas the binding points at the lower concentrations fall well below that curve. This result suggests that a cooperative process is involved in binding of rho to the RNA under these conditions. Since rho is an oligomeric protein that functions as a hexamer but readily dissociates into dimers when the salt concentration is increased (Finger and Richardson, 1982), this cooperativity process could reflect some aspect of the concentration dependence of this dissociation-association reaction on binding. In an experiment performed by Marie Pak in this laboratory, it was found that the extent of dissociation of free rho was about the same in 0.15 M KGl as in 0.15 M KC1. Whatever the reason for this qualitative difference in the binding, these results indicate that rho does not bind the lacZ 217 transcript with significantly higher affinity in 0.15 M KGl than in 0.05 M KC1. If anything, the 0.15 M KGl disfavors binding at low concentrations of rho.

Very similar results were obtained with the lacZ 176 transcript (data not shown). General conclusions of these filter binding experiments using homogeneous RNA molecules were also confirmed by comparison of yields of transcripts of various sizes retained as a function of rho concentration in the different solutions when rho was added to a mixture of the various lacZ transcripts terminated at several different stop points (Zou, 1990).

Potassium Glutamate Enhances Rho ATPase with LacZ RNA as Cofactor—A sensitive measure of the multiple interactions between rho and RNA that are related to termination is the rate of ATP hydrolysis catalyzed by rho. With the lacZ 217 RNA, at saturation, the rate was three times higher in the presence of 0.15 M potassium glutamate than in the presence of 0.05 M KC1 (Fig. 9). With lacZ 176 RNA the difference in rates under the same set of comparable conditions was 2-fold higher (data not shown). Thus, an enhancement that is seen at the level of termination correlates with a strong enhancement in the cofactor activity of the isolated RNA. Since an enhancement was not detected with the RNA-binding reaction, it must be related to interactions between rho and RNA that are subsequent to the primary binding reaction, interactions that are coupled to hydrolysis of ATP and that are responsible for termination of transcription.

Possible Role of Glutamate as a Magnesium Ion Buffer—Previous studies have shown that the rate of ATP hydrolysis catalyzed by rho with an mRNA as cofactor is decreased by

\[ f_s = e \cdot \frac{[\text{rho}]K_a}{1 + [\text{rho}]K_a} \]

where $f_s$ is the % of RNA retained, $K_a$ is the binding affinity constant, $\rho$ the molar concentration of rho, and $e$ the retention efficiency (in %) for the filters. The values of $e$ and $K_a$ for the two curves are presented in the text.

![Fig. 8. Binding of rho to lacZ-217 RNA in 0.05 M KCl and 0.15 M KGl. 25 fmol of $^{32}$P-labeled lacZ-217 RNA (16,000 cpm) was incubated with rho at the indicated concentrations at 37°C for 5 min in 100 μl of the respective binding solutions. The amounts of RNA retained on filters were determined as described under "Experimental Procedures." •, in 0.05 M KCl; △, in 0.15 M KGl. The curves are based on the rho excess binding isotherm equation (Faus and Richardson, 1989).]
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changes in conditions that stabilize RNA secondary structure (Richardson and Macy, 1981). Part of this evidence was the finding that rates of ATP hydrolysis with mRNAs as cofactors decrease sharply when the magnesium concentration is increased above the value needed to form the active substrate, Mg-ATP. This presumably occurs because magnesium ions strongly stabilize RNA secondary structure. However, the glutamate dianion chelates Mg$^{2+}$ ions. Thus, KGlu could be allowing higher rates of ATP hydrolysis in 4 mM total magnesium salt because it buffers the concentration of free Mg$^{2+}$ ions. To test this concept, the ATPase activity with lacZ 217 RNA was measured as a function of the concentration of magnesium salt added to reaction mixtures containing either 0.05 M KC1 or 0.15 M KGlu. The results (Fig. 10) show that the optimum concentration for magnesium salt is shifted from below 0.5 mM in the presence of 0.05 M KC1 to 4 mM in the presence of 0.15 M KGlu, thus indicating that, in some way, the KGlu has altered greatly the involvement of magnesium ions in the ATPase activity in lacZ RNA.

To demonstrate that this “activation” of ATP hydrolysis with KGlu is a general characteristic of transcripts from rho-dependent terminators whose function is enhanced by glutamate, we measured the rate of ATP hydrolysis with lambda-cro RNA as a function of magnesium salt concentration in reaction mixtures containing 0.05 M KC1 and 0.15 M KGlu. This RNA serves as a model for the nascent RNA that is terminated by rho action $\lambda$-trI, a relatively strong terminator. Again the magnesium optima in the two solutions are very different (Fig. 11). In this case, however, the optimum with 0.15 M KGlu is broad; there is little difference in ATP hydrolysis between 1.5 and 5 mM, while with 0.05 M KC1 it is relatively sharp, as with the lacZ RNA. Another difference is that the rates of ATP hydrolysis/unit of rho under the most favorable conditions in each salt is about six times higher with lambda-cro RNA than with the lacZ 217 RNA. This higher intrinsic rate could be the major reason why rho acts much more efficiently at $\lambda$-trI than at the lacZ RD220 terminator.

In the reaction mixtures used for studying termination during transcription of the lambda-cro gene DNA fragment, the magnesium salt concentration was 4 mM. With that level of magnesium salt, the rate of ATP hydrolysis with lambda-cro RNA was nearly 7 times higher with 0.15 M KGlu than with 0.05 M KC1. This difference in ATP hydrolysis again correlates well with the relative efficiencies of rho action with the two types of salts. Previously, Faus and Richardson (1989) found that the rates of ATP hydrolysis with lambda-cro RNA were nearly the same in 0.05 M KC1 and 0.15 M KGlu. However, those rates were measured in reaction mixtures that contained 1 mM magnesium salt. Fig. 11B shows that the activity profile curves for the two sets of conditions cross at that point.

The extent to which glutamate is buffering the concentration of free magnesium ions can, in principle, be calculated from the published stability constants for the magnesium-glutamate complex and the pK values for glutamic acid. However, since we found a discrepancy between a published value for the stability constant (Lumb and Martell, 1953) and the value in the database for a computer program that we used to calculate the free magnesium content in the complex mixture (Westall et al., 1976), we resorted to a dye-binding method to measure the actual concentration of [Mg$^{2+}$]free in our reaction mixtures (O’Sullivan and Smithers, 1979). Our results confirmed that glutamate does significantly reduce the level of free magnesium ions; for example, the values obtained with 5 mM total magnesium salt were 4.3 and 2.0 mM in the reaction mixtures containing 1 mM ATP and either 0.05 M KC1 or 0.15 M KGlu, respectively. A computer fit of the measured values with glutamate present is consistent with a...
stability constant for the Mg\textsuperscript{2+}-Glu\textsuperscript{2} complex of 316 M\textsuperscript{-1}, a value that is right between the two discrepant values. Using the data from the measured concentration of free magnesium ions, the data presented in Fig. 11A is replotted as a function of [Mg\textsuperscript{2+}]\textsubscript{free} (Fig. 11B). Since the curves are not coincident for the two salt solutions, we conclude that KGlu is affecting some other aspect of the magnesium ion dependence of the reaction besides the concentration of free magnesium ions.

**DISCUSSION**

The results presented in this paper demonstrate that rho factor can act with high efficiency at several different terminators during transcription *in vitro* in reaction mixtures that contain from 0.1 to 0.2 M potassium glutamate as the major electrolyte. This finding resolves two major discrepancies concerning the function of rho in cell-free systems. One discrepancy was the inability of rho to act with physiologically relevant levels of K\textsuperscript{+} ions in reaction mixtures containing KCl as the major electrolyte. In *E. coli*, the intracellular K\textsuperscript{+} ion concentration is about 0.2 M (Epstein and Schultz, 1965). Rho is virtually inactive with nearly every terminator tested in reaction mixtures that contained 0.2 M KCl. We have now shown that it is fully active with several terminators in mixtures containing 0.2 M KGlu. The other discrepancy was its inability to act at the intragenic lacZ terminators with a high enough efficiency during transcription of lacZ DNA *in vitro* to account for the termination that occurs when the same DNA is transcribed *in vivo* in the absence of coupled translation. The *in vivo* termination efficiency, measured with a galactokinase expression vector system, was about 97% (Ruteshouser and Richardson, 1989). However, in reaction mixtures containing 0.05 M KCl, which is the optimal level for that salt, the cumulative termination for the same DNA segment *in vitro* was about 50% (Table I, see also Ruteshouser and Richardson, 1989). In contrast, the cumulative termination efficiency in the reaction mixture with 0.15 M KGlu was 97% (Table I). Thus, the efficiency is matched with this physiological electrolyte, not with KCl.

Our interpretation of how KGlu affects termination is based on the studies of the rates of ATP hydrolysis catalyzed by rho with isolated transcripts as cofactors. There is considerable evidence that the ability of rho to terminate transcription is dependent on its actions on RNA that are coupled to the hydrolysis of ATP (Galluppi *et al.*, 1976; Sharp and Platt, 1984). These actions presumably are the driving forces that cause dissociation of the RNA from the transcription complex (Richardson and Conaway, 1980; Brennan *et al.*, 1987). How rho actually dissociates the RNA is still unclear, but it is dependent on binding of rho to a specific region in the RNA (Faus and Richardson, 1990), called a *rut* site, that is primarily single-stranded (Chen *et al.*, 1986) and on an extension of the contacts of rho with the RNA toward the 3' end. Since the extension of contacts with the RNA may also be necessarily with single-stranded segments, the process of extension would involve the breaking of base pairs that are part of the RNA secondary and tertiary structures. Magnesium ions bind to RNA molecules (Lynch and Schimmel, 1974) and stabilize secondary and tertiary structure interactions in polynucleotides (Dove and Davidson, 1962) presumably because of the bridging effect of the divalent cation in holding two strands together. tRNA molecules are known to have both high and low affinity sites for Mg\textsuperscript{2+}. Since the dissociation constants for the low affinity sites are in the 1 mM range, changes in the concentration of free Mg\textsuperscript{2+} near that value should greatly influence the extent of binding to those sites and thus the stability of the secondary structure. With KCl as the main electrolyte counterion, the rate of ATP hydrolysis by rho with *lacZ* or *λ-cro* RNA as cofactors is strongly inhibited by increasing the magnesium concentration above the optimum level of about 0.5 mM. This inhibition is likely to be a consequence of stabilization of secondary and tertiary structures caused by binding of magnesium ions to low affinity sites on the *cro* and *lacZ* transcripts. Essentially, we are arguing that the energetics of breaking the noncovalent bonds in RNA controls the rate-limiting step of ATP hydrolysis by rho.

With *λ-cro* RNA, the rate of ATP hydrolysis at the respective optimum for magnesium salt in the two different ionic environments was the same (Fig. 11). This suggests that the change of salt environment from 0.05 M KCl to 0.15 M KGlu affects primarily the way in which the magnesium salts are able to influence the structure of *λ-cro* RNA or the way the RNA interacts with rho factor. Although the ability of glutamate to buffer the concentration of free magnesium ions is contributing to this influence, the fact that the curves for ATPase activity as a function of [Mg\textsuperscript{2+}]\textsubscript{free} in the two salt solutions do not coincide indicates that the KGlu is also influencing how the free magnesium ions interact with the RNA or rho. Monovalent cations compete with magnesium ions in binding to nucleic acids (Record *et al.*, 1976), and the concentration of free potassium ions is very different in the two solutions, being 0.05 M with KCl and 0.15 M with KGlu. If it is assumed that a bound Mg\textsuperscript{2+} ion stabilizes RNA base pairing better than the K\textsuperscript{+} ions that would displace it, this cation competition mechanism could facilitate actions of rho on RNA that are coupled to ATP hydrolysis.

A critical property of KGlu that allows it to act effectively as a Mg\textsuperscript{2+} ion buffer and as a modulator of the binding of magnesium ions to RNA or rho is the relatively weak affinity of the glutamate anion for cationic groups in proteins (Leirmo *et al.*, 1987). This is important because the binding of rho to RNA is stabilized by salt bonds (Faus and Richardson, 1989), and the strength of those bonds is dependent upon the relative affinity of the counterions for the groups that form those bonds. As was shown by the binding studies, rho is able to bind with adequate affinity to *lacZ* 217 RNA in the presence of 0.15 M KGlu to activate ATP hydrolysis. These results with the *lacZ* RNA are fully consistent with the extensive measurements that have been made of the binding affinity of rho for *λ-cro* RNA as a function of different concentrations of KCl and KGlu (Faus and Richardson, 1989); *Kc* for that reaction was about the same in 0.15 M KGlu as in 0.05 M KCl and 0.05 M KGlu. However, the affinity of rho for *λ-cro* RNA was 10-fold lower in 0.15 M KCl than in 0.05 M KCl. This means that KGlu can be used at a concentration that is sufficient for it to have good Mg\textsuperscript{2+} ion buffering capacity and for it to modulate magnesium ion binding by cation competition without compromising the stability of the rho-RNA complex. The buffering capacity also provides a reservoir of magnesium atoms that can bind to the nucleoside triphosphate substrates for RNA polymerase. Thus, glutamate has the right combination of two critical properties: a relatively weak affinity for cationic groups in proteins and a weak but significant affinity for magnesium ions. These properties are also shared to some extent with the other salts that also enhance rho function, such as potassium aspartate, potassium D-glutamate and sodium glutamate. On the other hand, acetate, which also binds to cationic groups relatively weakly but does not bind magnesium ions substantially, allowed rho to function at a counterion concentration of 0.15 M but only to the level obtained with 0.05 M KCl.

Even though rho can make functional interactions with
nascent lacZ RNA and λ-cro RNA that lead to termination of the synthesis of those transcripts at their respective terminators, the strength and nature of the interactions with the two RNAs are very different. The lacZ-217 RNA binds rho less well than does λ-cro RNA (about 3.7-fold lower affinity in 0.15 M KGlu), and activates rho ATPase less well (about 6-fold with 4 mM magnesium acetate in 0.15 M KGlu). The two RNAs also have very different magnesium salt sensitivity as cofactors for rho ATPase in 0.15 M KGlu (cf. Figs. 10 and 11). Rho action in transcription termination is a dynamic process, and the efficiency of rho action depends on how rapidly rho can bind to the nascent RNA and how rapidly it can act on the RNA (Richardson, 1990). The former property is reflected indirectly in the binding affinity, while the latter is reflected in the ATP hydrolysis reaction with excess RNA. As a consequence of the weaker binding and poorer ATPase activation with the lacZ transcript, much higher concentrations of rho are needed to achieve efficient termination at the lacZ terminators than at λ-tR. This is consistent with previous observations made using mutational variants of X-tRI (Faus and Richardson, 1989) that the efficiency of rho action at a specific terminator depends very much on how well rho can interact with the nascent RNA.

E. coli contains a complex mixture of anions in its cytoplasm. Although glutamate is often a major component in this mixture, it may account for only about 15% of the anionic charge under most conditions of growth (Richey et al., 1987), the rest being from inorganic anions such as phosphate and sulfate and other metabolites, such as lactate, acetate, aspartate, citrate, and succinate. Although no precise measurements are available, there is evidence that chloride is not a major intracellular constituent (Stork et al., 1977). The fact that relatively low levels of chloride prevent rho from acting with its in vivo efficiency is further evidence that chloride is not a normal intracellular anion. Our results concerning the effect of salts on rho-dependent transcription terminators reinforce the conclusion of Leirmo et al. (1987) that KGlu is particularly well suited for mimicking the in vivo environment for transcription. It is certainly more suitable than KCI as an electrolyte component for studies of protein-nucleic acid interactions, and because of its Mg$^{2+}$ ion buffering capacity, it may be particularly suited for studies with RNA.

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