Mutations in the ATP-Binding Domain of *Escherichia coli* rho Factor Affect Transcription Termination In Vivo

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Five mutant rho proteins, representing alterations at three different locations in the *Escherichia coli* rho gene that affect ATP hydrolytic activity but not RNA binding, were examined in vivo for function at the rho-dependent IS2 and bacteriophage lambda *f*~ρ~1~*~ terminators. The altered amino acids in rho are located at highly conserved residues near the B1 and B4 strands of the hydrophobic ATP-binding pocket that is structurally similar to the F~1~-type ATPases and adenylate kinase. The RNA-dependent ATPase activities of the mutant rho proteins were previously shown to range from undetectable to a twofold increase over wild-type rho in vitro. Analysis of these proteins within the environment of the cell confirmed that transcription termination in vivo is indeed related to the ability of rho factor to properly hydrolyze nucleoside triphosphates, as would be predicted from results in vitro. The relative efficiency of termination at lambda *f*~ρ~1~, as judged by lambda N~w~ plating efficiency and by suppression of polarity of IS2 upstream of *galK*, was closely linked to the level of RNA-dependent ATPase activity observed in vitro for each protein. Moreover, the termination efficiency of four of the altered rho proteins at IS2 and lambda *f*~ρ~1~*~ in vivo corresponded directly to the effect of these mutations on rho function at the *E. coli* trp t' terminator in vitro. We conclude that determinations of rho function in vitro accurately reflect its behavior in intracellular termination events.

The *Escherichia coli* transcription termination factor rho is required for correct RNA 3' end formation at specific, rho-dependent sites (28, 29, 35). rho protein was discovered and characterized as a termination and release factor in an in vitro transcription reaction using bacteriophage lambda DNA (32). This essential cellular protein consists of identical subunits of 419 amino acids that assemble into a homenamer in solution (12, 26, 27). rho binding to RNA, with particularly high affinity for cytokine-containing synthetic ribopolynucleotides (14, 19, 30), serves to activate its ATPase activity (20). In the presence of its RNA substrate, rho hydrolyzes ATP (15, 18, 19) to ADP and P~i~. This activity can be observed in vitro, uncoupled from transcription termination, but is required for efficient termination (15, 31) and RNA-DNA helicase activity in vitro (2, 7). The release of RNA from an RNA-DNA hybrid is presumably used in vivo to facilitate release of nascent RNA transcripts from their DNA templates (31, 34). A current comprehensive review of the properties and activities of rho protein is available elsewhere (10).

In an analysis of rho protein structure and function, we have shown that the RNA-binding domain resides within the first 151 amino acids of the polypeptide (9). An ATP-binding domain was proposed to exist in the region extending approximately from amino acids 160 to 360 of rho, based on sequence and predicted structural homologies with other nucleotide-binding proteins (9). In addition, the novel ATP affinity analog, pyridoxal-5'-diphospho-5'-adenosine, uniquely labels Lys-181 within the highly conserved ATP-binding region (8). Site-directed mutagenesis of specific residues within this domain indicated that Lys-181 and Lys-184 are both involved in modulating ATPase activity, probably by interactions required for catalysis. The alteration of Asp-265 to asparagine revealed that this highly conserved residue is also necessary for ATPase activity. Each of these altered proteins was characterized extensively in vitro and shown to be affected in ATPase and helicase activity, as well as transcription terminating ability at the *trp* t' terminator (7), but not in ability to bind RNA. The effect of these alterations in the ATP-binding site on the behavior of rho in transcription termination in vivo was not determined.

We examine here the transcription termination ability of the mutant rho factors in vivo, using two independent assay systems. The first is based on the bacteriophage lambda termination-antitermination system. In lambda, expression of genes required for lytic growth is dependent on the lambda N protein (for a review, see reference 13). This protein is an antitermination factor that promotes readthrough of the terminators *t*~ρ~1~, *t*~ρ~3~*~ and *t*~ρ~2~*~. The late genes necessary for lytic development are preceded by *t*~ρ~1~ and *f*~ρ~3 (Fig. 1). Lambda N7N55cI26 (hereafter referred to as lambda N~w~) makes no functional N protein, excluding antitermination at the rho-dependent lambda *f*~ρ~1~ terminator. This mutant is unable to grow in a host producing wild-type rho factor unless it also carries amber suppressors. If rho factor is defective for termination, then the lambda N~w~ strain grows lysotypically. In this case, N protein is not required for readthrough of lambda *f*~ρ~1~; since rho is not effectively terminating at these sites. The plating efficiency of lambda N~w~ was used to test rho function in vivo, since a host deficient in rho function allows growth of the mutant phage.

Other laboratories have used similar systems to characterize rho mutants in vivo. A polarity suppressor mutant of *E. coli*, rho-15(Ts) was shown to allow growth of lambda N7, lambda N500, and lambda N7N53, whereas no propagation of these phage strains was allowed by the wild-type host (4). N-independent early leftward and rightward transcription of the lambda genome in the absence of the N gene product was also demonstrated for several *nita* (rho) mutants by using a variety of lambda phages with amber mutations in N (16, 17).

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FIG. 1. Partial genetic map of bacteriophage lambda. The direction of transcription from the \( p_L \) and \( p_R \) promoters is indicated by horizontal arrows. The termination sites where \( N \) protein acts as an antitermination factor are indicated by vertical arrows. \( N \) is transcribed from \( p_L \) as part of the early genes.

The second system used to characterize altered rho proteins involved determining the amount of readthrough of the rho-dependent IS2 terminator (6) when located upstream of the gal operon. The strong polar effects of insertion elements (IS1 and IS2) appear to be due to the introduction of translational stop codons in all three reading frames, exposing termination sites located distal to or within the insertion. Polarity at IS1 and IS2 can be suppressed by mutations in rho (5). Galactokinase expression in the presence of mutant rho protein was used as a measure of termination deficiency when IS2 precedes the structural gal genes, providing a second independent analysis of rho function in vivo at a different rho-dependent region.

Using the lambda \( t_{K1} \) and IS2 terminators, we compare here the relative effectiveness of five rho proteins altered within the ATP-binding domain for four different site-directed mutations in rho (the fifth protein was insoluble in vitro). A direct correlation exists between (i) the results of analyses in vitro of ATPase activity and termination at trp t' and (ii) the behavior in vivo at the rho-dependent, lambda \( t_{K1} \) terminator, as well as the suppression of polarity at IS1 and IS2 elements. These findings imply that the ability of rho to hydrolyze nucleoside triphosphates is essential for its activity in vivo and that the combined analysis from various assays for rho function in vitro provide an accurate reflection of its behavior within the cell at several different rho-dependent terminators.

MATERIALS AND METHODS

Strains and phage. Bacterial strains and phage used are listed in Table 1. Bacterial culture medium was made according to Miller (24) or Maniatis et al. (22). Restriction enzymes, calf intestine phosphatase, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Inc., and used under the conditions recommended. DNA fragments used in ligations were electrophoresed from agarose gels into Tris-acetate buffer (22).

Construction of \( E. coli \) strain DW319rho115. Strain DW319 can be used for phenotypic selection of polar mutations (11). As a control for the lambda \( N^* \) plating experiments, the rho-115 gene was transduced into the DW319 strain background. This was accomplished using P1 phage transductions performed both according to Miller (24) and as follows. Lysates were prepared by infecting an \( E. coli \) culture, grown in LB medium containing 5 mM CaCl2, with P1 vir at mid-log phase of growth. Lysis becomes apparent after 3 to 4 h. A few drops of chloroform were added to the culture, followed by centrifugation at 10,000 \( \times g \) for 5 min. The supernatant was stored over chloroform at 4°C as P1 vir[donor]. The recipient strain was grown to mid-log phase in LB medium containing 5 mM CaCl2, and 0.4 ml was infected with 0.1 ml of several dilutions of P1 vir[donor] for 25 min at 37°C. Sodium citrate was added to chelate the Ca\(^{2+} \) and prevent further infection. The cells were pelleted, suspended in 0.1 ml of M9 salts, and spread on selective plates. The plates were incubated at 37°C overnight. Transductants were then tested for other selectable markers as appropriate. The parent strain used to generate P1 vir lysates for transduction of the rho-115 allele was RVNrho115 ilv::Tn10. The rho gene is cotransducible with the ilv (isoleucine/valine) operon (3), and thus transductants were selected for tetracycline resistance (Tn10) and checked for inability to grow on glucose minimal medium unless supplemented with isoleucine and valine. In addition, the strain carrying rho-115 forms dark red colonies on MacConkey-melibiose at 42°C. DW319 and DW319rho115 were used as the wild-type and rho- controls for all lambda \( N^* \) plating experiments, as well as the host recipients for plasmids carrying each of the mutant rho alleles from site-directed mutagenesis.

Plating of lambda \( N^* \) phage. \( E. coli \) cells were grown to saturation in 10 ml of LB medium (22) supplemented to 0.2% maltose and, when appropriate, with ampicillin (100 \( \mu \)g/ml). The cultures were pelleted, and suspended in 4 ml of 0.01 M MgSO4, and stored at 4°C. A 15-\( \mu \)l sample of this culture was diluted into 85 \( \mu \)l of 0.01 M MgSO4 for plating, mixed with 100 \( \mu \)l of lambda N7N55cI26 of various dilutions, and incubated at 37°C for 20 min. The mixture was added to 3.5 ml of LB top agar (22) at 48°C, plated onto LB plates or onto LB medium plus ampicillin (100 \( \mu \)g/ml), and incubated at 39°C for 12 to 16 h.

Construction of the pCIR plasmids and protein induction. The gene coding for cI857 was excised from pMS7 on a
transcribed from the lambda pl promoter. This promoter remains repressed by c1857, expressed from lambda prm on the same plasmid. Because of the thermolabile repressor, repression is dependent on growth at temperatures of 32°C or less, and rho expression can be induced by growth at 37 to 42°C.

To construct a cloning vehicle for analysis of the rho mutations in vivo, the pCIR plasmid was restriction digested with EcoRV to remove a 1,800-base-pair fragment containing all but the first 144 base pairs of the rho gene and the 3'-flanking sequences; religation at the EcoRV sites generated the pCIR plasmid (Fig. 2B). The mutated rho alleles, KQ181, KQ184, KQ181/184, DN265, and DV265 (Fig. 3), had been cloned into pCIR. Each of these mutations had been characterized in vitro on protein overexpressed in the p39-AS plasmid (7). The 1,800-base-pair EcoRV fragment (Fig. 2B) was electroeluted from 1% agarose for each mutant derivative of p39-AS, ligated into pCIR and then linearized by EcoRV digestion, and treated with calf intestinal phosphatase to prevent reclosure of the vector. The resulting plasmids are identical to pCIR except that they carry one of the mutated rho genes (Fig. 3).

Each plasmid was transformed into two different host strains, DW319 and DW319rho115. Since the analysis of function in vivo was dependent on expression of the mutant rho factors from their pCIR derivatives, induction of rho was checked in each of the transformed strains. Cultures (10 ml) of DW319pCIR and DW319rho115pCIR were grown in LB medium plus ampicillin (100 μg/ml) at 30°C until reaching an optical density at 550 nm of approximately 0.5. The cultures were then shifted to incubation at 39°C for 0 to 22 h. At various times, cells were pelleted by centrifugation, treated with sodium dodecyl sulfate (SDS)-urea sample buffer, and analyzed on 0.1% SDS–10% polyacrylamide gels. In each case, the mutant rho proteins were induced to levels similar to those shown in Fig. 4.

**GalK assays.** Galactokinase (GalK) activity (23) was determined as a differential rate of synthesis, i.e., the slope of the line generated when enzyme activity is plotted versus cell growth (A600). For measurement of termination efficiency at IS2 upstream of galK, cultures were grown overnight at 30°C in LB medium plus ampicillin (100 μg/ml), diluted 1:500 in LB medium plus d-fucose (10 mM) and ampicillin (100 μg/ml), and grown for 2 h at 30°C. The cultures were then shifted to 42°C, and each strain was sampled three to four times within two doublings during exponential growth.

**RESULTS**

We report here on the behavior in vivo of several mutations in the ATP-binding domain of rho factor (Fig. 3) generated by site-directed mutagenesis (7), by assaying for their effects on lambda N' plating. This first required constructing an expression system that could be used in the DW319 strain background. Our original rho-overproducing vector p39-AS (25) had limited versatility because rho expression was governed by the lambda repressor gene, cI, carried in the host strain, and overexpression of rho was induced by nalidixic acid. This in itself was lethal to the cell, as well as being impossible to regulate in terms of obtaining a range of rho levels. We therefore devised an alternative expression system that could be maintained in almost any strain, with the potential for variable degrees of induction. A thermolabile version of lambda repressor, c1857, was used by Schmidt and Chamberlin (33) to regulate expression of
the *nusA* gene in plasmid pMS7 (Fig. 2A). We constructed the pCIR series of plasmids, in which *rho* is repressed by the action of c1857, carried by the plasmid itself, at *p*1 (Fig. 2). Each mutant *rho* gene was inserted into this plasmid, and expression of the altered protein was induced by temperature shift from 30 to 42°C. These plasmids can be maintained in many strains.

The use of strain DW319 provides a color phenotype system for a qualitative analysis of rho function. DW319 carries a *lac* polar mutation consisting of an IS1 insertion element near the 3′ end of the *lacZ* gene. Expression of the next gene, *lacY* (coding for lactose permease), is thus severely reduced by the polar effect of the IS1 element. IS1 contains a rho-dependent terminator; therefore, polarity in this system is due to rho-dependent termination within IS1. The expression of *lacY*, and therefore the efficiency of termination in IS1, can be evaluated by demanding growth that is dependent on expression of *lacY*. With melibiose as the carbon source, uptake is normally by way of the melibiase permease. This permease, however, is inactive at 42°C, and cells can utilize this sugar only if an alternative pathway is available, via the lactose permease. On MacConkey-melibiose agar, fermentation of the carbohydrate source results in a red colony color, and cells with a normal rho factor grown at 42°C are white, since *lacY* is not expressed and melibiose cannot be imported. If the strain carries a mutant rho factor, allowing *lacY* expression at some level due to relief of polarity, the colonies at 42°C will be red. Table 2 shows colony color phenotypes at 42°C for the strains carrying mutant rho factors. In general, the colonies harboring defective rho proteins were light pink to pink, whereas those with efficient rho factors were white. This finding suggests that the ability of mutant rho factors to suppress polarity at IS1 coincides with termination efficiency at lambda *tR1* as discussed below. In this test, DW319(pCIR-KQ181/184) grew poorly at 42°C.

Like the DW319 strains, the DW319rh0115 strains were evaluated for colony color phenotype on MacConkey-melibiose plates at 42°C. The results shown in Table 3 indicate agreement between loss of ability to terminate at lambda *tR1* and ability to suppress the polarity introduced by IS1. Effective termination in this system resulted in white colonies, whereas lack of termination produced red colonies. The rescue of the DW319rh0115 red phenotype was clearly shown for pCIR, pCIR-KQ181, and pCIR-KQ184, with partial rescue by pCIR-ND265 and pCIR-DV265.

**Lambda N" plating.** The ability to plate the mutant lambda strain, lambda N7/N55ci26 (lambda N"), was used to measure the relative termination efficiency of each rho protein as a termination factor at the rho-dependent terminator lambda *tR1*. Evaluation of the effectiveness of rho factor was completed by using two strain backgrounds, DW319 and DW319rh0115. DW319 carries the wild type *rho* gene in a single copy on the chromosome. This strain was transformed with the pCIR derivatives of each mutated *rho*. Induction of rho expression by temperature upshift in this system resulted in very high levels of rho protein from the plasmid copy of rho, effectively diluting the wild-type rho protein produced from the chromosomal copy of rho. The concentration of rho in the cells remained high over the entire period of lambda infection. Figure 4 shows rho levels up to 22 h after temperature upshift. Cultures were infected with lambda N" phage and plated at 39°C to ensure rho induction.

![Diagram](image)

**Fig. 3.** Map of amino acid substitutions in rho protein. Long arrows indicate the locations of the RNA- and ATP-binding domains. Numbers above the diagram denote the positions chosen for oligonucleotide site-directed mutagenesis. The mutations made are listed below, with arrows showing the substitution made at each site. The nomenclature used to identify each mutation is also listed, using conventional single-letter amino acid codes. The original amino acid is followed by the substitution and the location within the rho polypeptide.
TABLE 3. Characterization of altered proteins in DW319rho115

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Efficiency of plating lambda N\textsuperscript{+} at 39°C (a)</th>
<th>Colony color on MacConkey-melibiose(b)</th>
<th>Function in vitro(c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.04</td>
<td>Red</td>
<td>NA</td>
</tr>
<tr>
<td>pBRrho</td>
<td>0.96</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>pCIR (wild type)</td>
<td>0.022</td>
<td>Light pink</td>
<td>++</td>
</tr>
<tr>
<td>pCIR-KQ181</td>
<td>0.033</td>
<td>Light pink</td>
<td>+</td>
</tr>
<tr>
<td>pCIR-KQ184</td>
<td>0.005</td>
<td>White</td>
<td>+ + +</td>
</tr>
<tr>
<td>pCIR-KQ181/184</td>
<td>1.10</td>
<td>Red (poor growth)</td>
<td>–</td>
</tr>
<tr>
<td>pCIR-DN265</td>
<td>0.044</td>
<td>Dark pink</td>
<td>+/–</td>
</tr>
<tr>
<td>pCIR-DV265</td>
<td>0.051</td>
<td>Dark pink</td>
<td>Insoluble</td>
</tr>
</tbody>
</table>

\(a\) Normalized to the phage titer obtained with an amber suppressor strain. Two to sixteen independent plating assays were used to calculate the values shown.

\(b\) Incubation at 42°C.

\(c\) In vitro analyses were described by Dombroski et al. (7). The relative function is based on ATPase activity. ++ +++, Highest observed activity; –, no activity obtained with an amber suppressor strain; NA, not assayed.

DW319 plates lambda N\textsuperscript{+} very poorly, since it carries wild-type rho, which terminates transcription at lambda tR1, preventing lytic growth. The introduction of a defective rho factor into this strain increases the lambda N\textsuperscript{+} plating efficiency.

Lambda N\textsuperscript{+} was plated on several DW319pCIR strains, and the plating efficiencies obtained at 39°C were tabulated. Table 2 gives the results for lambda N\textsuperscript{+} plating with DW319 as the host strain. Relative rho function in vitro is given in Table 2 in the last column as discussed in Dombroski et al. (7). DW319 with no plasmid lambda N\textsuperscript{+} very poorly. A control plasmid, pBRrho, is a derivative of pBR322 in which rho is cloned into the PuvII and HindIII sites of the plasmid but there is no promoter for rho expression. This plasmid serves as a control for the effect of a plasmid that is not expressing rho on lambda N\textsuperscript{+} plating. The presence of this plasmid had no effect on the lambda N\textsuperscript{+} plating efficiency of DW319. The remaining plasmids were all pCIR derivatives in which rho expression is induced from the p1 promoter by temperature upshift. DW319 carrying pCIR (wild-type rho factor) showed the same plating efficiency as the host strain alone, as expected. Mutations to glutamine in the ATP-binding site of rho at Lys-181 (KQ181) and Lys-184 (KQ184) did have varying effects on lambda N\textsuperscript{+} plating. Plasmid pCIR-KQ181 caused an approximately 10-fold increase in lambda N\textsuperscript{+} plating efficiency over the wild-type rho factor. This mutation was shown to have a fivefold-lower ATPase activity in vitro. Thus, it behaved as a defective rho in vivo as well as in vitro. Plasmid pCIR-KQ181/184 carries a very defective rho protein, as assayed in vitro; when assayed in DW319 for lambda N\textsuperscript{+} plating, it caused a 230-fold increase in plating efficiency, indicating that it is also very defective for transcription termination in vivo. The KQ184 mutant was shown to have about a twofold better ATPase activity in vitro, and in vivo it also showed a slightly better ability to terminate, since it gave the lowest lambda N\textsuperscript{+} plating efficiency. The effectiveness of transcription termination in vitro at tR1 for this mutant was in fact slightly diminished, but this may be a peculiarity of the assay, since termination in vivo at tR1 agrees with ATPase and helicase function in vitro. Nonetheless, each of the lysine mutations demonstrated very good correlation between the in vitro and in vivo analyses of function.

The mutations to asparagine (DN265) and valine (DV265) generated at Asp-265 were also within the ATP-binding site of rho. DN265 was shown to be quite defective in vitro, with a 125-fold decrease in ATPase activity. Characterization of DV265 in vitro is impossible because the protein was insoluble. The analysis of this mutation in vivo is therefore the only indication of its effect on rho function. Characterization of these mutations in vivo gave better than expected termination phenotypes. Both were no more defective than KQ181 for termination at lambda tR1 in the lambda N\textsuperscript{+} plating test, but DN265 was decidedly poorer in all in vitro measurements. Possibly the in vitro results are misleading because of protein instability, as addressed in more detail in Discussion. With the exception of DN265 and DV265, the plating of lambda N\textsuperscript{+} appears to be a good indicator of rho function in vivo and correlates well with in vitro ATPase, helicase, and termination assays. A parallel set of lambda N\textsuperscript{+} plating experiments was conducted, using DW319rho115 as the host strain. The rationale here was to evaluate the ability of each mutation to rescue the severely defective termination phenotype of DW319rho115. In this case, the question asked is, “How well (rather than how poorly) do these mutants function in termination?” A reversal of the highly effective lambda N\textsuperscript{+} plating ability of DW319rho115 was sought for the same set of plasmids used in the DW319 host system. Table 3 shows the results of lambda N\textsuperscript{+} plating in DW319rho115. The host strain carrying rho-115 alone demonstrated very good plating efficiency, unlike its parent carrying wild-type rho. This phenotype was not altered by the presence of pBRrho115, as expected. When pCIR (wild-type rho) was introduced, the plating efficiency dropped by at least 45-fold; thus, wild-type rho can rescue the rho-115 defect. This base-line level of plaque formation differs from that obtained in the DW319 strain and may reflect either competition between the rho proteins for binding to tR1 or the mixing of wild-type and mutant rho subunits. Likewise, KQ181 rescued rho-115 but not to the extent of wild type; this result is consistent with its reduced in vivo activity. The KQ181/184 protein, inactive in vitro, did not rescue the rho-115 defect. The increased in vitro activity of KQ184, however, displayed a reduction in plating on the rho-115 strain to levels equivalent to or better than those for the parent strain, paralleling its increased activity in vitro. Again, the phenotype of lambda N\textsuperscript{+} plating

FIG. 4. rho induction during lambda N\textsuperscript{+} plating. Strain DW319 carrying pCIR was grown in LB medium plus ampicillin (100 µg/ml) to an optical density at 550 nm of 0.5 at 30°C. The culture was shifted to 42°C, and samples were removed at 0, 3, 6, and 22 h after induction. Cells were centrifuged, suspended in SDS-urea sample buffer (6 M urea, 5% 2-mercaptoethanol, 2% SDS, 10% [vol/vol] glycerol, 125 mM Tris hydrochloride [pH 6.8]), and heated for 3 min at 90°C. These samples were loaded onto a 0.1% SDS–10% polyacrylamide gel and stained with Coomassie brilliant blue. Lanes: 1, total protein before induction; 2 to 4, total protein at 3, 6, and 22 h. The position of rho is indicated.
efficiency in vivo agrees with the results of protein analysis in vitro as well as with the results of lambda N" plating using DW319 as the host. The same experiment was done with pCIR-DN265 and pCIR-DV265. In both cases, the defective rho-115 phenotype was rescued but to a lesser extent than for wild type, KQ181, or KQ184. As was the case for DW319 plating, the DN265 mutation functioned better than expected on the basis of its performance in vitro.

Termination activity at IS2. E. coli strain 7478 carries an IS2 insertion sequence in the leader region of the gal operon. IS2 contains a rho-dependent termination region, rendering this strain Gal-. Expression of the gal operon in this system provides a means for quantitating rho function in vivo, since the same strain (strain 7479) carrying rho-15 instead of wild-type rho on the chromosome is Gal+. Each of the pCIR derivatives harboring a mutant rho gene was transformed into strain 7478, and GalK assays were used as a measure of readthrough at IS2. Cultures were grown at 30°C and shifted to 42°C to induce rho expression, and samples were taken at various time points for GalK analysis. Table 4 shows the rates of GalK synthesis at 42°C. Strain 7478 without a plasmid as well as with pBRrho, pCIR, and pCIR-KQ184 resulted in a differential rate of about 1.0. The severely defective KQ181/184 mutant had a rate of 17.8, whereas the less defective KQ181, DN265, and DV265 proteins resulted in rates of 6.5, 3.2, and 2.8, respectively. These rates provide an indication of the relative effectiveness of each altered rho for termination at yet another rho-dependent site in vivo. The values obtained coincide well with the in vitro functional assays for each protein and with the results of lambda N" plating. Figure 5 shows results of rho induction during GalK assays.

Table 4 also shows the results of growth rate measurements for doubling time and colony color phenotype on MacConkey-galactose plates. The presence of various pCIR derivatives had significant effects on the growth rate of the host cell. In particular, DN265, DV265, and KQ181/184 slowed the rate of doubling by 3- to 12-fold in L broth with ampicillin at 42°C. This decrease in growth rate reinforces the essential nature of rho activity for normal cell function and the significance of poisoning by mutants defective in ATPase activity. Similarly, the colony colors displayed on MacConkey-galactose plates at 39°C (cells grew very poorly on this medium at 42°C) were in agreement with quantitative measurements of function. The most defective rho proteins generated a red colony phenotype, as seen for KQ181/184 and for strain 7479, which carries rho-15 on the chromosome. Intermediately defective rho genes gave pink colonies, and normal rho gave a white phenotype. The data presented in Table 4 are consistent with the results of in vitro analysis and with in vivo analysis in strains DW319 and DW319rho115.

DISCUSSION

The characterization of site-directed mutations in the rho factor ATP-binding domain previously revealed interesting alterations in behavior on the basis of analysis of ATPase, helicase, and transcription termination activities in vitro (7). Yet the pleiotropic effects of rho mutations are difficult to predict from the results of analysis in vitro. If, as commonly assumed, the termination function of rho in vivo requires ATPase activity, then the behavior of these mutations (which have no effect on RNA binding [7]) in vivo should reflect their behavior in vitro. We have examined these mutations in vivo by several criteria: (i) ability to support lambda N" growth in DW319, (ii) ability to reduce lambda N" plating in a rho mutant host, and (iii) polarity suppression at an IS2 element upstream of galK. The consistency of behavior at a variety of terminators examined in vitro and in vivo provides satisfying evidence that rho action is similar at different rho-dependent sites.

The analysis of altered rho factors in vivo required construction of a new expression system. Introduction of the altered rho genes into the pCIR vector resulted in plasmids that (i) carry a copy of the thermolabile repressor, c1857, as well as the rho gene that must be repressed, (ii) can be maintained in virtually any E. coli strain background, and (iii) can be induced to overexpress rho by growth at temper-
atures of between 37 and 42°C. These plasmids are notable for their ease of maintenance and retention of cell viability even upon rho induction.

The determination of lambda N' plating ability using two complementary approaches provided a measure of termination efficiency at lambda tR1. Two different host strains were used as recipients for the various pCIR expression plasmids carrying mutant rho alleles. Strain DW319 harbors a copy of the wild-type rho gene on the chromosome and is normal with respect to rho function. This strain is unable to plate lambda N', since rho terminates transcription normally at lambda tR1, preventing lytic growth of the phage. Altered rho genes introduced on pCIR and induced to express mutant proteins by temperature upshift were assayed for defective-ness of termination and thus an increase in lambda N' plating efficiency. The results of lambda N' plating for the KQ181, KQ184, and KQ181/184 mutants were in very good agreement with the prior results of ATPase, helicase, and transcription termination assays in vitro at the E. coli trp t' rho-dependent terminator. The significance of this agree-ment takes on even greater importance when one considers that two different terminators were used: trp t' from the tryptophan operon of E. coli for in vitro assays and tR1 from the lambda genome for in vivo characterization. Experiments conducted with the DN265 mutant, however, demon-strated that this mutation was not as defective as indicated by in vitro analysis. There are two plausible explanations for this apparent discrepancy. First, it is possible that this altered protein loses activity during the purification steps, and therefore its behavior in vitro is not representative of its behavior in vivo. Alternatively, the level of function witnessed in vitro, although low, is sufficient for fairly effective termination in vivo. The latter explanation is less attractive on the basis of the very good correlation obtained between lambda N' plating and in vitro analysis for the mutations at Lys-181 and Lys-184.

The second strain used for lambda N' plating experiments was DW319rho115. This strain carries the rho-115 allele and is defective in transcription termination. DW319rho115 was also used as a host for each of the pCIR derivatives in lambda N' plating experiments. The use of DW319rho115 allowed a determination of the relative ability of each protein to rescue the effect of rho-115, indicating its degree of normal termination ability. The same mutations used in DW319 were then tested for their ability to rescue the wild lambda N' plating efficiency of DW319rho115. Again, the results obtained for KQ181, KQ184, and KQ181/184 as well as for wild-type rho agreed with the in vitro analysis and with their behavior in strain DW319. The rho factors that functioned well in vitro were able to rescue the rho-115 defect, and the level of reversal was correlated with relative in vitro function. Again, the DN265 and DV265 mutants were able to terminate transcription better than expected on the basis of their behavior in vitro.

Colony color phenotype determined in DW319 and DW319rho115 on MacConkey-melibiose at rho induction temperatures agrees with the quantitative analyses and provides a third terminator (IS1) in which rho function can be assessed. Tests of colony color provide only a qualitative indication of rho function in vivo. Lambda N' plating is a more quantitative approach, but it is the result of a complicated series of events required for lambda development and is only indirect. Therefore, a second quantitative technique was invoked to provide additional support for the termination behavior of mutant rho factors in vivo. A reporter gene placed downstream of a regulatory site is a common method for evaluating a protein that acts at that site. Strain 7478 contains an IS2 insertion element in the gal operon within the leader sequence. Since IS2 carries a rho-dependent termination region, the strain is Gal'+ as long as rho is functioning properly. The phenomenon of polarity has generally been attributed to rho-mediated termination of transcription within insertion elements, and thus rho mutants are able to relieve polarity introduced by insertion (5, 6). The system provided by strain 7478 allowed quantitation of rho termination efficiency at IS2 for each of the rho mutations discussed above. Rates of GalK synthesis for cells induced for rho expression were in very good agreement with all the previously discussed data for termination efficiency of the altered rho factors. In addition, colony color phenotypes on MacConkey-galactose plates indicated that rho function within the cellular environment parallels function determined by testing in vitro.

In summary, the quantitation of in vivo transcription termination at rho-dependent sites can be successfully accomplished by using lambda N' plating assays as well as reporter gene expression downstream of IS2. The results of this analysis are very reproducible and correlate with the various tests of rho function in vitro. The behavior of rho factor at trp t', lambda tR1, IS1, and IS2, representing four different rho-dependent termination sites, was consistent as well. The mutations at Lys-181 and Lys-184 of rho all behaved as expected in this termination assay, as judged by their behavior in vitro. The ability of rho to hydrolyze ATP and to act as a helicase and a transcription termination factor in vitro appears to be directly related to its performance in intracellular termination events.

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LITERATURE CITED


