Rho-dependent Transcriptional Polarity in the ilvGMEDA Operon of Wild-type Escherichia coli K12*

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It has been generally accepted that transcriptional polarity in prokaryotic systems is due to an uncoupling of translation and transcription which unmasks latent rho-dependent termination sites in a polycistronic messenger RNA. In this report, we identify and characterize rho-dependent termination sites responsible for transcriptional polarity in the ilvGMEDA operon of wild-type Escherichia coli K12. The ilvG gene in the wild-type E. coli K12 ilvGMEDA operon contains a frameshift site which results in termination of translation in the middle of the gene. Mutations have been characterized which restore the reading frame of this gene. In addition to allowing full-length expression of the ilvG product, these mutations cause a 3–4-fold elevation in the expression of the operon distal genes. This transcriptional polarity effect on operon distal genes also has been shown to be relieved by rho suppressor mutations. We have used in vitro transcription experiments to identify rho-dependent transcriptional polarity sites downstream of the frameshift site in the ilvG gene. Three tandem rho-dependent sites have been located in the ilv'GM' gene region using transcription reactions containing linear or supercoiled plasmid DNA templates. Accumulatively, these rho-dependent termination sites account for about 80% in vitro transcription termination, which is in agreement with the in vivo measurements of transcriptional polarity on operon distal gene expression. These transcriptional experiments provide in vitro confirmation for the latent rho-dependent termination site model of transcriptional polarity.

In prokaryotic systems, the premature termination of translation of a polycistronic messenger RNA can reduce transcription of operon distal genes. This phenomenon, transcriptional polarity, is the result of any mutation that causes termination of translation within a proximal gene of an operon (for review, see Refs. 1–4). Mutations in the transcriptional termination factor rho have been shown to alleviate this polar effect on distal gene expression (5–8). In order to explain transcriptional polarity, it has been proposed that rho-dependent transcriptional termination sites exist in operons, but these sites are not recognized by rho factor when transcription and translation are tightly coupled. It is further presumed that the uncoupling of transcription and translation by premature translational termination unmasks these previously latent rho-dependent termination sites and that termination at these sites explains transcriptional polarity (1–3).

An example of transcriptional polarity is seen in the ilvGMEDA operon of wild-type Escherichia coli K12. The first two genes of this operon, ilvG and ilvM, encode the large and small subunits, respectively, of acetohydroxy-acid synthase II. This enzyme, which catalyzes the first step of the parallel pathway for the biosynthesis of the branched-chain amino acids, isoleucine, valine and leucine, is one of three isozymes present in E. coli (9, 10). One of the differences distinguishing these isozymes is the sensitivity to end-product inhibition by valine. Acetohydroxy-acid synthases I and III are inhibited by valine, whereas the acetohydroxy-acid synthase II isozyme is not. Lawther et al. (11) demonstrated that the ilvG gene in wild-type E. coli K12 contains a frameshift site which results in termination of translation approximately in the middle of the gene. This explains why E. coli K12 is growth-inhibited by exogenous valine, that is, the remaining two active acetohydroxy-acid synthase isozymes are both inhibited by valine, which blocks the synthesis of isoleucine. A secondary explanation for growth inhibition in the presence of valine is provided by the action of the sulfonylurea herbicide sulfometuron methyl, which inhibits the growth of bacterial cells by inhibiting acetohydroxy-acid synthase enzymatic activity (12). This inhibition results in a toxic accumulation of the acetohydroxy-acid synthase enzyme substrate α-ketobutyrate (13). By analogy, the end-product inhibition by exogenous valine of the two acetohydroxy-acid synthase isozymes (acetohydroxy-acid synthases I and III) expressed in E. coli K12 could result in a toxic accumulation of this pathway intermediate.

Several E. coli K12 mutations have been isolated which are resistant to growth inhibition by exogenous valine. In each case which has been characterized, there is a 1-base pair deletion or a 2-base pair insertion within a 10-base pair region preceding the frameshift site in the ilvG gene (11, 14). Each mutation allows for the full-length expression of the ilvG product and therefore functional acetohydroxy-acid synthase II activity. In the valine-resistant strains, the specific activities of transaminase B and threonine deaminase, the products of the operon distal ilvE and ilvA genes, respectively, are elevated 3–4-fold compared to wild-type E. coli K12 (15, 16). Isogenic strains containing rho mutations also show an increase in distal gene expression as compared to the wild-type E. coli K12 strain (15, 16). Therefore, both types of mutations appear to relieve classical transcriptional polarity effects on distal gene expression in the ilvGMEDA operon.

In this report, additional aspects of transcriptional polarity in the ilvGMEDA operon are examined. In vivo analyses of
the effects of the valine-resistant ilvG468 mutation and rho221 suppressor mutation in isogenic strains show that whereas either mutation alone increases the expression of the distal ilvE gene product, the two mutations together do not have an additive effect. This is consistent with both mutations relieving a common transcriptional polarity event. In vitro transcription experiments using DNA templates containing the tac promoter fused to a restriction fragment downstream of the ilvG frameshift polarity suppression site identify two tandem rho-dependent termination sites encoded in this region. These sites, like other rho-dependent termination sites, are sensitive to different salt concentrations and require sequences upstream of the site of transcription termination. The transcription experiments provide in vitro confirmation for the latent rho-dependent termination site model of transcriptional polarity.

**Experimental Procedures**

**Materials**—Radiochemicals were purchased from Amersham Corp. All enzymes were from Boehringer Mannheim or New England Biolabs. Rho factor was a gift from Terry Platt (Department of Biochemistry, University of Rochester Medical Center, Rochester, NY).

**Cloning**—The col21 strain from the transcription B assay were as follows: T3I-4-4, trpE9829(Am), trpA9761(Am), thi-1(17); T3I-4-490, trpE9829(Am), trpA9761(Am), thi-1, rho221 (16); and T3I-4-590, trpE9829(Am), trpA9761(Am), thi-1, ilvG468 (16). Strain IH-57, trpE9829(Am), trpA9761(Am), thi-1, rho221, ilvG468, was constructed by P1 transduction of strain T3I-4-590 with T3I-4-490 as donor. Selection was for valine resistance and growth in the presence of indole as a source of tryptophan (16).

**Plasmid Constructions**—An 807-bp PvuII-SalI restriction endonuclease fragment containing the ilu'G' gene region (Fig. 1) was isolated from plasmid pH28 (11) and inserted into the SalI sites of plasmid pUC8 (18). From this plasmid construct, a 255-bp EcoRI-HindIII fragment containing the ilu'G' insert and the flanking polylinker region of plasmid pUC8 was isolated and inserted into the EcoRI site of HindIII sites of plasmid pKK223-3 (19). These two restriction sites in plasmid pKK223-3 are located between the tac promoter and the rnrB TTT2 terminator sites. The resulting plasmid pPS1 contains the truncated ilu'G' gene region of the insert adjacent to the tac promoter and the ilu'M' region adjacent to the rnrB TTT2 terminators. Plasmids pPS30 and pPS31 were constructed in a similar fashion. A 504-bp AluI-SalI restriction endonuclease fragment containing the ilvG468 gene region (Fig. 1) was isolated from plasmid pH29 and inserted into the SalI site of plasmid pUC8. From plasmid constructs with both orientations of the insert, 531-bp EcoRI-HindIII restriction endonuclease fragment containing the ilu'G' gene insert and the flanking polylinker region of plasmid pUC8 was inserted into the SalI sites of plasmid pKK223-3. The resulting plasmid pPS30 contains the truncated ilu'G' gene region of the insert adjacent to the tac promoter and the ilu'M' region adjacent to the rnrB TTT2 terminators. Plasmid pPS31 contains the insert in the opposite orientation.

**Transaminase B Assay**—Transaminase B assays were performed by the method of Duggan and Wechsler (20). Bacterial cultures were grown in M63 minimal medium. (21) to 100 klett units and collected by centrifugation. The cell pellet from a 2-ml culture was resuspended in a 1.0-m1 solution of 100 mM KPO4 (pH 7.5) and disrupted by sonication. After centrifugation, a sample of the clear cell lysate of a-ketogluturate and 50 pmol of valine and incubated at 37 °C for 15 min. The reaction was terminated by the addition of 1.0 ml of 0.5% 2,4-dinitrophenyhydrazine in 2 M HCl. The sample was incubated for 15 min at room temperature, 2.0 ml of toluene was added, and the mixture was vortexed for 3 min. The aqueous and organic phases were separated by centrifugation, and 1.0 ml of the toluene layer was removed and mixed with 5.0 ml of 10% Na2CO3. Following centrifugation, 2.0 ml of the Na2CO3 layer was removed and mixed with 2.0 ml of 1 N HCl. The absorbance was measured at 540 nm following a 10-min incubation at room temperature. The protein concentration of the cell extracts was determined by the method of Lowry et al. (22).

**Purification of DNA**—Cesium chloride band-purified plasmid DNA was prepared by standard methods (23). Linear DNA restriction fragments were separated by electrophoresis on 5% polyacrylamide gels, 0.5% acrylamide, 0.1 M Tris borate (pH 8.3) and 1 mM EDTA (TBE). The polyacrylamide gels were stained with ethidium bromide, and restriction fragment bands were dissected from the gels, dissolved in 20% β-mercaptoethanol, and purified on DEAE columns.

**In Vitro Transcription**—Purified supercoiled plasmid DNA was used as a template in the in vitro transcription reactions. A 9-μl solution containing 0.1 pmol of DNA template, 40 mM Tris acetate (pH 7.9), 4 mM magnesium acetate, 0.1 mM dithiothreitol, 0.1 mM EDTA, 200 μM ATP, 20 μM CTP, 10 μM GTP, and 0.1 pmol of [α-32P]GTP (800 Ci/mmol), and 50 units/ml RNA polymerase (0.9 pmol) was incubated for several minutes at 37 °C. The KCl concentration was 50 mM unless otherwise noted. Where indicated, 0.1 μg of rho factor (0.4 pmol) was added per reaction. The transcription reactions were initiated with the addition of 200 μM CTP and 200 μM UTP. After a 20-min incubation at 37 °C, a phenol/ chloroform extraction was performed, and 15 μg of E. coli tRNA was added to the sample, followed by ethanol precipitation. The dried pellets were dissolved in a solution of 4 M urea, 0.05% sodium dodecyl sulfate, 0.125% bromphenol blue, and 0.125% xylene cyanol blue, heated for 1-2 min at 95 °C, and analyzed by electrophoresis on a 6% polyacrylamide denaturing gel containing 8 M urea buffered with TBE. The gel was fixed in a solution of 10% methanol and 10% acetic acid, dried under vacuum and exposed to Kodak XRP-5 film. Plasmid pUC19 (24) digested with restriction enzymes or uncut was filled at the recessed 3'-end with [α-32P]GTP using the Klenow fragment of E. coli DNA polymerase I (23). The 32P-labeled DNA fragments were used as markers on the polyacrylamide/urea gels. These markers were calibrated with RNA transcripts of known length to account for electrophoretic differences between RNA and DNA molecules. The percentage of transcription termination at each site was quantitated by dissecting transcript bands from the polyacrylamide/urea gels and measuring radioactivity by liquid scintillation spectroscopy. These measurements were normalized for the guanine content of each transcript.

**Computer Analysis of RNA Secondary Structure**—Analysis of RNA secondary structure of the ilv'GM' gene region was conducted using the FOLD program (25) contained in the University of Wisconsin Genetics Computer Group program library (26). To estimate the temporal nature of RNA folding in the nascent transcript, overlapping nucleotide sequences of 900 and 100 nucleotides in length were examined for secondary structure using RNA and DNA molecules. The percentage of transcription termination at each site was quantitated by dissecting transcript bands from the polyacrylamide/urea gels and measuring radioactivity by liquid scintillation spectroscopy. These measurements were normalized for the guanine content of each transcript.

**RESULTS**

The iluG gene in the ilvGMEDA operon of wild-type E. coli K12 contains a frameshift site which results in termination of the encoded product approximately in the middle of the gene (11, 14). Several mutants have been characterized which produce a full-length iluG product. One example is the strain T3I-4-590, which contains the ilvG468 mutation, a single adenine deletion upstream of the frameshift site (Fig. 1) (14). In addition to restoring acetohydroxy-acid synthase II activity, this mutation results in a 5.4-fold increase in the operon. The stability of iluG gene expression compared to the isogenic wild-type E. coli K12 strain (Table 1). This polar effect on downstream gene expression is also alleviated by rho suppressor mutations. The rho221 mutation encodes a defective rho protein with a larger molecular weight than wild-type as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7). A wild-type E. coli K12 strain containing the rho221 mutation shows a 5.2-fold elevation in ilvE expression compared to the isogenic wild-type strain (Table 1). Although E. coli strains containing either mutation alone increase the ilvE-encoded transaminase B activity above that in the wild-type isogenic strain, the two mutations together in strain II-
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FIG. 1. Restriction endonuclease map of the ilvGMEDA operon of E. coli K12. The open bars indicate the extent of the coding region for each of the designated genes. Preceding the first gene of the operon, ilvG, the tandem in vitro promoters (P1P2), leader (e), and attenuator (a) are indicated. The restriction map is shown on the solid line. Below the restriction map of the operon is an enlargement of the region containing the 807-bp PvuII-SalI restriction fragment. The wavy line indicates transcription into this gene region. Three tandem rho-dependent termination sites are designated, and the location of each is listed relative to the start codon of the wild-type E. coli K12 is indicated. The ilvG468 mutation is a deletion of a single adenine residue just upstream of the wild-type stop codon. This mutation allows for the full-length expression of the ilvG product. The letter designations for restriction endonucleases are as follows: A, AluI; C, ClaI; E, EcoRI; H, HindIII; K, KpnI; P, PvuII; S, SalI; and X, XhoI.

TABLE I

| Strain | Relevant genotype | Specific activity transaminase B (ilvE)* | Ratio |
|--------|------------------|----------------------------------------|-------|
| T31-4-4 | Wild type        | 29                                     | 1     |
| T31-4-480 | rho221        | 151                                    | 5.2   |
| T31-4-590 | ilvG468       | 158                                    | 5.4   |
| IH-57   | rho221, ilvG468 | 163                                    | 5.6   |

*Transaminase B-specific activities are expressed in nanomoles/minute/milligram of protein.

57 do not increase the distal gene expression of the ilvGMEDA operon greater than that which is due to only one mutation (Table I). This is consistent with both mutations relieving a common transcriptional polarity event.

To characterize directly the effects of rho factor in transcriptional polarity, we performed in vitro transcription reactions using DNA templates containing the strong tac promoter fused to restriction fragments downstream of the ilvG frameshift site. An 807-bp PvuII-SalI restriction fragment containing the ilvG′ gene region (Fig. 1) was inserted into the polylinker region of plasmid pJSPl as template were also carried out in the presence of the tac promoter and the rrnB T1T2 termination sites. Since many rho-dependent termination sites have been shown to be salt-sensitive in in vitro transcription reactions (2, 27, 28), we used a 25–150 mM KCl concentration range in transcription reactions containing this linear BamHI-HindIII restriction fragment template. As shown in the autoradiogram in Fig. 2 (lanes 1–8), the titration in KCl concentration affected the number and intensity of the rho-dependent terminated transcripts. In the presence of rho factor at 50 mM KCl (lane 3), the 865-nucleotide read-through transcript indicated by the arrowhead (transcript synthesized from the tac promoter to the end of the linear template) is faint, whereas at least three smaller transcripts are visible. These transcripts are not seen in transcription reactions in the absence of rho factor (lanes 1 and 8). As the KCl concentration is increased, the smaller transcripts are no longer visible, whereas the rho-dependent terminated transcripts labeled 1–3 are retained (lanes 4 and 5). At a concentration of 150 mM KCl, the rho-dependent transcripts are diminished, and only the read-through transcript is visible (lane 7).

In vitro transcription reactions using the supercoiled plasmid pJSPl as template were also carried out in the presence of transcription termination sites located in the ilvG′ gene region. A 1080-bp BamHI-HindIII restriction fragment containing the tac promoter fused to the ilvG′ gene region was used as a linear DNA template in in vitro transcription reactions in the presence and absence of rho factor (lanes 1–8). In lanes 1 and 8 are the in vitro transcription products from reactions containing linear DNA template and transcription mixtures with 50 (lane 1) or 100 (lane 8) mM KCl in the absence of rho factor. In lanes 2–7 are the products from transcription reactions containing linear DNA template, rho factor, and transcription mixtures with an increasing concentration of KCl. The KCl concentration in each transcription reaction lane was as follows: lane 2, 25 mM; lane 3, 50 mM; lane 4, 75 mM; lane 5, 100 mM; lane 6, 125 mM; and lane 7, 150 mM. The 865-nucleotide read-through transcript indicated by the arrowhead is the result of transcription from the tac promoter to the end of the linear template. Transcription bands resulting from termination at the major rho-dependent sites, 1–3, are indicated. In lanes 9 and 10 are the products of in vitro transcription reactions containing the supercoiled plasmid pJSPl. Plasmid pJSPl contains the ilvG′ gene region adjacent to the tac promoter. Transcription reaction mixtures contained 50 mM KCl in the presence (lane 9) or absence (lane 10) of rho factor. The 1110-nucleotide transcript band indicated by the arrowhead is the result of transcription from the tac promoter terminating at the rrnB T1 site.
and absence of rho factor (Fig. 2, lanes 9 and 10, and Fig. 3, lanes 3 and 4). In this supercoiled template reaction, RNA polymerase initiates transcription at the tac promoter, and any transcripts which read through the ilu'GM' insert terminate transcription at the downstream rho-independent rrnB T1T2 sites. An autoradiogram of products from the in vitro transcription reactions performed at 50 mM KCl in the presence of rho factor shows three areas of rho-dependent terminated transcripts (Fig. 2, lane 5, and Fig. 3, lane 4). These three areas of transcripts co-migrate with the rho-dependent terminated transcripts seen in reactions using the linear restriction fragment as template (Fig. 2). In the absence of rho factor, a 1110-nucleotide transcript indicated by the arrowhead is visible (Fig. 2, lane 10, and Fig. 3, lane 3). This transcript is a result of rho-independent termination at the rrnB T1 site. A transcript about 175 nucleotides larger than the T1 terminated transcript is also detected (Fig. 3, lane 3). This larger transcript band, which has much less intensity, is a result of transcription termination at the rrnB T2 site (29).

In these in vitro transcription experiments using the tac promoter, we find that greater than 90% of the rho-independent transcription termination occurs at the rrnB T1 site. In vitro transcription reactions containing the parental plasmid pKK223-3 show only a single rho-independent terminated transcript (Fig. 3, lanes 1 and 2). Therefore, at least three tandem rho-dependent termination sites, as defined by in vitro transcription experiments, are located in the ilu'GM' gene region of the iluGMEDA operon of E. coli K12.

The nucleotide sequence containing the three tandem rho-dependent termination sites is shown in Fig. 4. The centers of the more efficient termination sites 1 and 2 are located in the iluM gene, whereas the weakest site 3 is centered in the distal portion of the iluG gene. It is noted that termination sites 2 and 3 migrate as doublets on the polyacrylamide/urea gel, and the sites of termination indicated in Fig. 4 are located in the approximate center of the transcript doublet.

The efficiency of these four termination sites in the in vitro transcription reaction containing the supercoiled plasmid pJSPl template in the presence of rho factor was quantitated by dissecting the transcript bands from the polyacrylamide/urea gels and measuring radioactivity by liquid scintillation spectroscopy. About 23% of the transcripts read through the rho-dependent termination sites. This is in agreement with the transaminase B assay of transcriptional polarity, where there was a 5-fold difference between the wild-type T31-4-4 strain and the isogenic strain containing the rho22l suppressor mutation (Table I). The percentages of transcripts terminated at the three tandem sites show a gradient effect, with the greater termination at the downstream sites. These termination percentages are as follows: site 1, 37%; site 2, 24%; and site 3, 16%.

To determine how much upstream sequence is required for termination of transcription, the supercoiled plasmid and the three tandem rho-dependent termination sites are indicated in Fig. 4 are located in the ilu'GM' gene region.

**Fig. 3.** In vitro transcription analysis of rho-dependent transcription termination using supercoiled plasmid templates. Restriction endonuclease fragments containing portions of the ilu'GM' gene region were inserted between the tac promoter and the rrnB T1T2 terminators of plasmid pKK223-3 as described under "Experimental Procedures." In vitro transcription products from reactions using these supercoiled plasmid templates in reaction mixtures containing 50 mM KCl in the presence or absence of rho factor were analyzed by electrophoresis in polyacrylamide/urea gels. Supercoiled plasmid templates in each reaction lane are as follows: lanes 1 and 2, plasmid pKK223-3; lanes 3 and 4, plasmid pJSPl; lanes 5 and 6, plasmid pJSPl0; and lanes 7 and 8, plasmid pJSPl3. Rho factor was added to reactions in lanes 2, 4, 6, and 8. Read-through transcript bands which result from transcription from the tac promoter terminating at the rrnB T1 site are indicated by arrowheads along with the corresponding transcript site in nucleotides. Transcript bands resulting from transcriptional termination at the major rho-dependent sites, 1-3, are indicated.

**Fig. 4.** Nucleotide sequence of the iluG frameshift region in wild-type E. coli K12 and the three tandem rho-dependent transcriptional termination sites located in the ilu'GM' gene region. The wild-type E. coli K12 iluG stop codon is indicated at position 1254 relative to the in vitro transcriptional initiation site. The iluG648 mutation is a deletion of an adenosine residue at position 1250 (11, 14). This deletion results in the translation of the iluG product to position 1917. Several other mutations have been isolated which allow the full-length expression of the iluG product. For example, iluG2868 is an adenosine deletion at position 1245, iluG671 is a ribosylthymine deletion of position 1248, iluG8969 is a ribosylthymine deletion at position 1254, and iluG2896 is a TA insertion between positions 1251 and 1252 (14). The nucleotide sequence of the three tandem rho-dependent termination sites is underlined with the site number listed below. Nucleotide sequences containing a single mismatch with the consensus sequence CAATCAA are indicated in brackets. The iluG termination codon of the full-length iluG product is indicated with three asterisks. The overlapping iluM initiation codon is underlined. Upstream of the rho-dependent transcription termination sites at positions 1654-1761 are 10 cytidine residues spaced by 11 or 12 nucleotides. Each spaced cytidine residue is indicated by an asterisk below the nucleotide. Regions lacking predicted secondary structure are underlined with dotted lines.
rho-dependent termination at the three tandem sites, a 503-bp \textit{AclI-Sall} insertion fragment containing the \textit{ilu'GM'} region (Fig. 1) was inserted into plasmid pKK223-3 between the \textit{tac} promoter and the \textit{rrnB} T1-T2 termination sites. The resulting plasmid pJSP30 contains the restriction fragment oriented such that the truncated \textit{ilu'} gene region of the insert is adjacent to the \textit{tac} promoter, whereas plasmid pJSP31 contains the restriction fragment insert in the opposite orientation, with the truncated \textit{iluM'} gene region adjacent to the \textit{tac} promoter. In \textit{vitro} transcription reactions were performed using these supercoiled plasmid templates in the presence and absence of rho factor. An autoradiogram of the products resulting from the transcription reactions containing plasmid pJSP30 shows only two rho-dependent terminated transcripts (Fig. 3, lanes 5 and 6). These transcripts correspond to downstream termination sites 1 and 2. Thus, it would appear that greater than 290 nucleotides is required upstream of termination site 3 for rho factor to recognize this site of termination, whereas sequences 300 and 400 nucleotides upstream of terminations sites 2 and 1, respectively, are sufficient for rho-dependent activity. In \textit{vitro} transcription reactions containing plasmid pJSP31, which contains the \textit{ilu'GM'} insert in the opposite orientation, show transcripts which terminated only at the rho-independent \textit{rrnB} T1-T2 termination sites (Fig. 3, lanes 7 and 8). The major 520-nucleotide T1 terminated transcript is indicated by the arrowhead. This is consistent with rho factor requiring recognition sequences upstream of the termination sites for activity.

**DISCUSSION**

We have used \textit{in vitro} transcription experiments to understand the molecular basis of transcriptional polarity downstream of the \textit{iluG} frameshift site in the \textit{iluGMEDA} operon of wild type \textit{E. coli} K12. Three tandem rho-dependent termination sites have been detected in transcription reactions containing the supercoiled plasmid pJSPl template (Fig. 2). These sites in the 3’-end of the \textit{iluG} gene and the 5’-end of the \textit{iluM} gene are located between 615 and 740 bp downstream of the \textit{iluG} frameshift site (Figs. 1 and 4). These sites show a gradient effect in the percentage of \textit{in vitro} rho-dependent termination, with downstream termination site 1 accounting for the greatest percentage (37%) of transcription termination. Accumulatively, these rho-dependent termination sites account for about 80% of \textit{in vitro} transcription termination. This is in agreement with the \textit{in vivo} measurements of transcriptional polarity on operon distal gene expression, where the \textit{iluE}-encoded transaminase B activity increased about 5-fold in strains containing either the rho221 suppressor mutation or the \textit{iluG}68 mutation compared to the isogenic wild-type \textit{E. coli} K12 strain (Table I).

Currently, it is not certain to what extent the relative contribution of the \textit{in vitro} rho-dependent termination sites in the \textit{ilu'GM'} gene region reflects \textit{in vivo} termination. In \textit{vitro} transcription reactions using a linear DNA template containing the strong \textit{tac} promoter fused to this \textit{ilu'GM'} gene region showed that the number and intensity of the rho-dependent terminated transcripts were differentially affected by increasing concentrations of KCl. At 50 mM KCl, maximal rho activity was observed (Fig. 2). As the concentration of salt was increased (75–100 mM), the smaller molecular weight rho-dependent terminated transcripts were diminished; whereas at 125 mM KCl, the rho-dependent terminated transcripts 1–3 were similarly affected. Previous investigators (2, 27, 28) have noted that the \textit{in vitro} termination activity of rho factor is inhibited at high salt concentrations on many linear DNA templates, with a maximal activity generally observed at approximately 50 mM KCl. A proposed explanation for these observations is that RNA secondary structure, which is inhibitory to rho action, is stabilized by high ionic concentrations (2). It is interesting to note, however, that \textit{in vitro} transcription reactions using the supercoiled plasmid pJSPl at 50 mM KCl showed three tandem rho-dependent terminated transcripts (Figs. 2 and 3). The lower molecular weight rho-dependent terminated transcripts seen in transcription reactions using the linear DNA template at this concentration of KCl were not visible. The transcripts encoded from linear or supercoiled DNA template are identical; and thus, RNA secondary structure would not be expected to differ. An alternative explanation for the effects of increased salt concentrations on rho-dependent transcription termination may reflect a difference in energy required to release the nascent transcript. Perhaps, greater energy is required to release rho-dependent terminated products from supercoiled DNA templates than from linear templates. This is consistent with the observation that products from \textit{in vitro} transcription reactions containing the supercoiled plasmid pJSPl in the presence of rho factor are not affected by modest changes (50–100 mM) in KCl concentration. In any case, we interpret these results to indicate that products from \textit{in vitro} transcription reactions containing the supercoiled plasmid template may reflect more closely \textit{in vivo} rho-dependent termination activity.

The nucleotide sequences important for rho-directed termination are located upstream of the site of transcription termination. Deletion analyses of DNA sequences upstream of the rho-dependent \textit{trp} \textit{t’} termination site show that sequences 50–100 nucleotides upstream of this site are sufficient for \textit{in vivo} and \textit{in vitro} rho activity, regardless of the sequence content of the downstream termination site (30). In the four tandem \textit{ilvR} rho-dependent terminators, RNA sequences 90–130 nucleotides upstream of each termination site are critical for rho recognition (31). In this study, nucleotide sequences encoded upstream of the \textit{Alul} restriction site (Fig. 1) are critical for \textit{in vitro} rho-dependent termination at site 3 (Fig. 4). This indicates that sequences considerably further upstream of a rho-dependent termination site than previously seen (>290 nucleotides) are required for rho recognition at termination site 3. This sequence is located downstream of the \textit{iluG} termination codon in wild-type \textit{E. coli} K12 and would be free of translating ribosomes and available for rho factor recognition. Termination sites 1 and 2 are located in the \textit{iluM} coding region, about 75 and 25 nucleotides, respectively, downstream of the \textit{iluM} initiation codon. By analogy to the \textit{trp} \textit{t’} and \textit{Alul} \textit{rho}-dependent termination examples, both sites would require RNA sequences encoded in the upstream \textit{iluG} gene for rho activity (Fig. 4). Again, these sequences in strains containing the \textit{iluG} frameshift site would be free of translating ribosomes. It is possible that a ribosome initiating translation at the \textit{iluM} start codon could interfere with rho-directed termination at these major \textit{in vitro} termination sites. However, it has been proposed that the \textit{iluM} gene is transcriptionally coupled to the \textit{iluG} gene (32, 33). The \textit{iluG} termination codon overlaps the \textit{iluM} initiation codon by four nucleotides in \textit{E. coli} K12 (Fig. 4). This overlap is thought to ensure the equimolar expression of the large and small acetolactate synthase II subunits, encoded by the \textit{iluG} and \textit{iluM} genes, respectively. Therefore, premature translation termination in the \textit{iluG} gene is predicted to inhibit \textit{iluM} translation.

An intensive effort has been made to distinguish a consensus rho factor recognition sequence upstream of the transcription termination site (4, 34, 35). One hypothesis suggested that a stem-loop structure and the sequence CAAUCAA up-
stream of the AtR1 termination sites could signal rho-dependent transcription termination (34). However, in vitro transcription reactions containing a synthetic DNA template encoding this sequence failed to show detectable rho activity (34). More recent models (4, 55) have included the following characteristics for rho-binding sites on nascent RNA transcripts: 1) a region located within a few hundred nucleotides upstream of the rho-dependent termination site, 2) a region spanning 70–80 nucleotides in length, and 3) a sequence void of stable secondary structure. The importance of single-stranded RNA regions in rho recognition has recently been demonstrated by Chen et al. (36). DNA oligonucleotides complementary to two regions upstream of the termination site were shown to impede rho directed termination of the AtR1 transcript.

The three tandem rho-dependent termination sites in the ilu'GM' gene region share some of these structural features. As mentioned, the consensus sequence CAAUCAA is found adjacent to several rho-dependent termination sites (34). Interestingly, this sequence with a single mismatch at the fifth or sixth position is found adjacent to each of the three termination sites (Fig. 4). These are the only examples of this sequence with complete identity or a single mismatch found in the 807-bp PvuII-SalI restriction fragment which contains the ilu'GM' gene region. The sequence of the ilu'GM' region has also been examined for RNA secondary structure. Several regions have been identified which contain no dyad symmetry. These include sequences relative to the in vivo iluGMEDA transcriptional start site at positions 1358–1384, 1419–1434, 1468–1492; 1535–1547, 1592–1607, 1704–1726, 1856–1866, and 1918–1929 (Fig. 4) (33). Finally, it has been noted that certain rho-dependent termination regions, including a region putatively important in rho-dependent transcriptional polarity in the his operon in Salmonella typhimurium, contain a regular spacing of cytidines (12 ± 1) (4). It is postulated that these sequences could be involved in nucleating rho binding to its recognition site. In the distal portion of the iluG gene, upstream of the three tandem rho-dependent termination sites, there is a region which contains 10 cytidine residues spaced by 11 or 12 nucleotides. This spacing of cytidines is indicated by asterisks in Fig. 4. This 105-bp region of cytidine symmetry has 18.5% guanosine composition and overlaps a region containing no predicted secondary structure. Additionally, there are regions upstream of the Alul restriction site, which is shown by the deletion experiment to be important for rho-dependent termination at site 3, that contain spaced cytidines. Six cytidines spaced at regular intervals (12 ± 1) are identified between positions 1415–1476 and 1499–1557 (33).

In conclusion, we have used in vitro transcription experiments to characterize rho-dependent termination downstream of the iluG frameshift site in the iluGMEDA operon of wild-type E. coli K12. Three tandem rho-dependent termination sites have been identified using a supercoiled plasmid template. These results provide confirmatory evidence for the latent rho-dependent termination site model of transcriptional polarity in the iluGMEDA operon of E. coli K12, that is, premature translation termination in the iluG operon unmasks downstream rho-dependent termination sites, which results in decreased expression of the operon distal genes.

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