Evidence *in vivo* that the DEAD-box RNA helicase RhlB facilitates the degradation of ribosome-free mRNA by RNase E

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The RNA degradosome of *Escherichia coli* is a ribonucleolytic multienzyme complex containing RNase E, polynucleotide phosphorylase, RhlB, and enolase. Previous *in vitro* and *in vivo* work has shown that RhlB facilitates the exonucleolytic degradation of structured mRNA decay intermediates by polynucleotide phosphorylase in an ATPase-dependent reaction. Here, we show that deleting the gene encoding RhlB stabilizes a *lacZ* mRNA transcribed by bacteriophage T7 RNA polymerase. Deleting the gene encoding enolase has little if any effect. Other messages transcribed by T7 polymerase are also stabilized by Δ*rhlB*. The effect of point mutations inactivating RhlB is comparable with the effect of deleting the gene. Primer extension analysis of the *lacZ* message indicates that RhlB facilitates endoribonucleolytic cleavage by RNase E, demonstrating a functional interaction between the RNA helicase and the endoribonuclease. The possible physiological role of an RhlB-RNase E pathway and the mechanisms by which RhlB could facilitate RNase E cleavage are discussed.

DExD/H protein | enolase | polynucleotide phosphorylase | RNA degradosome

Instability is an intrinsic property of mRNA, permitting rapid response to changes in transcription and, in some situations, posttranscriptional control of gene expression. In *Escherichia coli*, the overall pathway for mRNA degradation is well established although much remains to be learned about controlling the stability of specific transcripts (1, 2). Messenger RNA is initially inactivated and fragmented by endoribonuclease cleavage. The fragments are then digested by exoribonucleases in a tightly coupled process. Intermediates in mRNA turnover are rapidly degraded and are thus generally difficult to detect except in mutant strains where a gene encoding a component of the degradation machinery has been disrupted. The RNA degradosome contains RNase E, polynucleotide phosphorylase (PNPase), RhlB, and enolase (3–5). RNase E is the principal endoribonuclease in mRNA degradation. It is a large multidomain protein with an N-terminal catalytic region and a C-terminal noncatalytic region containing RNA binding sites and the protein scaffold for degradosome assembly (6, 7). PNPase is one of the enzymes that degrade mRNA fragments produced by RNase E. The function of enolase, a glycolytic enzyme, in the RNA degradosome is not established although recent work suggests a role in the posttranscriptional regulation of the major glucose transporter, encoded by *ptsG*, involving a small regulatory RNA (8, 9). RhlB is a DEAD-box RNA helicase (10) that facilitates the degradation of structured mRNA decay intermediates by PNPase (5, 11, 12). This activity, requiring ATP hydrolysis, is believed to involve the local unwinding of structures that block PNPase (5, 13). Ribonucleolytic multienzyme complexes in other organisms have also been shown to interact with DEAD-box or related proteins (13, 14).

The DEAD-box proteins are a family of putative RNA helicases that generally have RNA-dependent ATPase activity *in vitro*. They contain a characteristic set of conserved sequence motifs that were discovered in a comparison between the eukaryotic translation initiation factor eIF4A and the *E. coli* protein SrmB (15). eIF4A is involved in loading the eukaryotic small ribosomal subunit onto mRNA (16). SrmB is a chaperone in the assembly of the large ribosomal subunit of *E. coli* (17). The DEAD-box proteins are now known to be part of the ubiquitous DExD/H-box family of helicases that participate in many RNA unwinding and remodeling reactions (18). The DExD/H-box proteins together with other RNA and DNA unwinding enzymes constitute a super family containing a structurally conserved ATPase domain with RecA-like architecture (19). This domain is likely to be a generic motor for the unwinding and remodeling of nucleic acids. RhlB and eIF4a, which are among the smallest members of the DExD/H-box family, contain little more than this conserved catalytic core. The DExD/H-box domain often associates with additional domains, either covalently in the same polypeptide or noncovalently within a multienzyme complex. RhlB and eIF4A are DExD/H-box helicases that require a protein partner for biological activity (6, 20).

Here, we have studied *in vivo* the role of RhlB and enolase in the degradation of a *lacZ* mRNA transcribed by bacteriophage T7 RNA polymerase (T7-*lacZ* mRNA). Because the phage polymerase is 8-fold faster than its *E. coli* counterpart, transcription outpaces translation, producing long stretches of ribosome-free mRNA. This message is exceptionally sensitive to inactivation and degradation by RNase E (21). The integrity of the noncatalytic region of RNase E, including the sites that bind RhlB and enolase, is necessary for this sensitivity (22, 23). Deleting the enolase gene has little if any effect on the T7-*lacZ* mRNA. Deleting the RhlB gene stabilizes the T7-*lacZ* mRNA, leading to a significant increase in β-galactosidase synthesis. We present evidence that RhlB is part of a specialized pathway involved in the degradation of ribosome-free mRNA by RNase E.

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Materials and Methods

The construction of ENS134-2, containing the *rne131* mutation, and ENS134-3, containing the *pnp*::Tn*5* mutation, has been described (22). SVK16, constructed here as a control, is isogenic to ENS134-3. SVK4 and SVK5 are derivatives of ENS134 and ENS134-2, respectively, in which the *rhlB* gene was disrupted (Δ*rhlB*) essentially as described (12). SVK62 and SVK63, containing the $rhlB^{DKAD}$ and $rhlB^{AAA}$ alleles, respectively, were constructed by the same replacement strategy. SVK17 is a derivative of SVK4 containing the *pnp*::Tn*5* mutation. TAZ2 is a derivative of ENS134 in which the *enolase* gene was disrupted by using the same strategy as for *rhlB*. The strains used in

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Abbreviations: PNPase, polynucleotide phosphorylase; IPTG, isopropyl β-D-thiogalactoside.

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Table 1. β-Galactosidase activity

Strain	Genotype	Activity (±SD, %)	Relative activity
ENS134	Wild type	1,670 (±9.4)	1.0
SVK4	Δ*rhlB*	7,090 (±9.4)	4.2
SVK16	*pnp*$^{-}$	5,430 (±7.1)	3.3
SVK17	Δ*rhlB pnp*$^{-}$	11,700 (±19)	7.0
ENS134-2	*rne131*	19,400 (±12)	11.6
SVK62	*rhlB*^{DKAD}	6,780 (±6.6)	4.1
SVK63	*rhlB*^{AAA}	6,710 (±9.6)	4.0

The results are the average of at least three independent measurements. Activities were measured as described (23). The SD as a percentage is in parentheses. Relative activity for each strain is normalized to wild type.

β-galactosidase assays with pEZ201 and pEZ206 (24) are derivatives of MC1061 that have been described (12). Liquid cultures were grown at 30°C in Mops medium (25) containing 0.4% glycerol, 0.2% casamino acids, and 0.1 mM isopropyl β-D-thiogalactoside (IPTG). For krox20 and engrailed expression, cells were grown to mid-log phase in LB medium containing 50 μg/ml ampicillin, induced with 1 mM IPTG and then incubated for 2 h. Preparation of protein extracts, immunoprecipitation, and Western blotting were as described (6, 26). RNA extractions and Northern blotting were as described (12). Northern blots were hybridized by using the HincII-KpnI fragment of pT7*lacZ-arg5* (27). For primer extension, the cells were cultured at 30°C without IPTG, then shifted to 42°C for 40 min. IPTG (0.1 mM) was added 10 min after the shift. RNA (4 μg) was mixed with ^{33}P-5′ end-labeled attcgcgtctggccttcctgt, dried, and suspended in 4 μl of annealing buffer (20 mM Tris·HCl, pH 7.5/80 mM KCl), heated to 85°C for 5 min, quenched on ice, and then incubated at 30°C for 30 min. Four microliters of RT buffer (75 mM Tris·HCl, pH 8.3/20 mM $MgCl_2$/4 mM DTT/0.8 mM each dNTP), containing 4 units of AMV reverse transcriptase (Promega), was added. Transcription was for 30 min at 42°C. A *lacZ* sequence was generated by using pT7*lacZ-arg5*, the same primer, and a Thermosequenase kit (Amersham Pharmacia).

Results

Deletion of the *rhlB* Gene Stabilizes the T7-*lacZ* mRNA. The ENS134 strain contains a chromosomally encoded *lacZ* gene that is transcribed by a chromosomally encoded bacteriophage T7 RNA polymerase. This system is under the control of the *lac* repressor and requires IPTG for induction. The level of synthesis of β-galactosidase in this strain can be used as an indicator of the stability of the *lacZ* message (21, 22). We will refer to this message as the T7-*lacZ* mRNA although it differs from authentic *lacZ* mRNA only due to its synthesis by T7 RNA polymerase. When the *rhlB* gene was deleted, 4.2-fold more β-galactosidase was produced (Table 1). To further examine this effect, we analyzed the amount of T7-*lacZ* mRNA by Northern blotting using a 5′-end probe (Fig. 1*a*). On this blot, the same quantities of RNA were loaded, and the quality of the transfer was checked with a probe against 5S rRNA (data not shown). The 4.3-kb mRNA is the full-length transcript containing *lacZ*, *lacY*′ (a fragment of *lacY*), and a tRNA (Fig. 1*c*). RNase E cleaves the 4.3-kb mRNA between *lacZ* and *lacY*′ to produce the 3.2-kb monocistronic *lacZ* transcript. The prominent small RNA in the lower half of the blot (Fig. 1*a*, asterisk) corresponds to the 5′-end fragments of the *lacZ* mRNA (27). They were not detected with an internal probe (data not shown). Lanes 1 and 2 in Fig. 1*a* show that the signal depends on IPTG. Comparison of lanes 3 and 4 shows that there is more T7-*lacZ* mRNA in the Δ*rhlB* strain. The effect on mRNA levels could be due to either increased transcription or increased mRNA stability. Because the tRNA

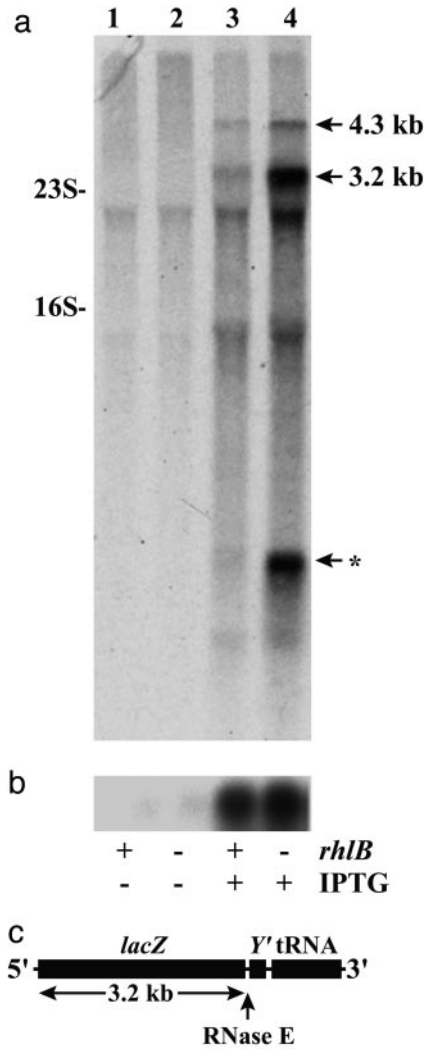

Fig. 1. Northern blot. (*a*) Total RNA from ENS134 (wild type) and SVK4 (Δ*rhlB*) was separated on a 1.5% agarose formaldehyde gel, transferred, and hybridized with a 5′ *lacZ* probe. In lanes 1 and 2, T7-*lacZ* was not induced. In lanes 3 and 4, the strains were cultivated continuously in the presence of IPTG. The position of the 4.3-kb primary transcript, the 3.2-kb monocistronic lacZ transcript, and a 5′ end fragment (asterisk) is indicated to the right of the panel. (*b*) The filter was stripped and hybridized with a probe against the arg5 tRNA. (*c*) Diagram of the 4.3-kb T7-*lacZ* transcript indicating the intercistronic RNase E processing site involved in the formation of the 3.2-kb monocistronic *lacZ* transcript (not drawn to scale).

processed from the T7-*lacZ* transcript is stable, it can be used as a reporter for transcription (21, 22, 27). The filter in Fig. 1*a* was stripped and rehybridized with a probe against the tRNA (Fig. 1*b*). Quantification showed a small, 1.3-fold increase in the tRNA in the Δ*rhlB* strain. Quantification of the 3.2-kb monocistronic mRNA and normalization to the tRNA level showed a 3.6-fold increase. Although the signal is too close to the background to accurately quantify, the 4.3-kb transcript also seems to increase in the Δ*rhlB* strain based on visual inspection of the image. Thus, the principal effect of deleting *rhlB* is the stabilization of the T7-*lacZ* mRNA.

Deletion of the *eno* Gene Does Not Stabilize the T7-*lacZ* mRNA. Enolase, like RhlB, is a major component of the RNA degradosome, and the purified degradosome has enolase activity (4, 5). To test its role in expression of the T7-*lacZ* mRNA, the *eno* gene was disrupted. The media used for the measurements in

Table 2. β-Galactosidase activity

Strain	Genotype	Activity (±SD, %)	Relative activity
ENS134	Wild type	2,242 (±2.3)	1.0
TAZ2	Δ*eno*	1,877 (±7.1)	0.84
SVK4	Δ*rhlB*	9,238 (±2.6)	4.1

The results are the average of three independent measurements. Activities were measured as described (23). The SD as a percentage is in parentheses. Relative activity is normalized to wild type.

Table 2 contained glycerol (0.4%) and lactate (0.2%) because the Δ*eno* strain requires both for growth (28). As a control, the Δ*rhlB* strain grown under the same conditions has 4.1-fold more β-galactosidase activity, which is comparable with the result in Table 1. There is a small but significant reduction of β-galactosidase in the Δ*eno* strain (84% of wild type). This difference could be due to the disruption of glycolysis in the Δ*eno* strain, which grows slower than wild type. From this experiment, we conclude that Δ*eno* does not stabilize the T7-*lacZ* mRNA.

RhlB Acts Independently of PNPase in the Degradation of the T7-*lacZ* mRNA. Previous *in vitro* and *in vivo* work has shown that RhlB facilitates the exonucleolytic degradation of structured mRNA decay fragments by PNPase (5, 11, 12). Although the major pathway for the turnover of the T7-*lacZ* mRNA is RNase E-dependent, a secondary PNPase-dependent pathway has been described (22). This pathway could involve exonucleolytic degradation of the 3.2-kb monocistronic transcript (Fig. 1*c*). The overproduction of β-galactosidase in the *pnp*$^-$ strain is 3.3-fold, under the conditions used here (Table 1). When *pnp*$^-$ was combined with Δ*rhlB*, the β-galactosidase level increased 7.0-fold. This result is strikingly different from previous *in vivo* work on the degradation of mRNA decay intermediates, in which the disruption of the *pnp* gene was epistatic to Δ*rhlB* (12). Thus, the effect of Δ*rhlB* on the turnover of the T7-*lacZ* mRNA is independent of PNPase, suggesting that RhlB has another function in mRNA degradation that has not previously been characterized. For comparison, the effect of the previously described *rne131* mutation (22, 23), in which the entire noncatalytic region of RNase E is missing, is an 11.6-fold increase in β-galactosidase (Table 1). Taken together, these results suggest that there are at least three pathways involved in the inactivation of the T7-*lacZ* mRNA: a PNPase pathway that does not require RhlB, an RNase E pathway that does not require RhlB, and an RNase E pathway that requires RhlB.

Deletion of the *rhlB* Gene Impairs Cleavage of the T7-*lacZ* mRNA by RNase E. We examined RNase E cleavages in the 5′ end of the T7-*lacZ* mRNA by primer extension (Fig. 2). In this experiment, strains were grown at 30°C and then shifted to 42°C. T7-*lacZ* transcription was induced after the shift by the addition of IPTG. Lanes 2 and 3 in Fig. 2 show, respectively, the result with either the wild-type control or the *rne1* temperature-sensitive strain. Lanes 1 (wild-type) and 4 (*rne1*) are background controls in which IPTG was not added. The 5′ ends marked by the asterisks were detected in wild type, but absent in the *rne1* strain (i.e., no signal above the background control in lane 4), showing that their production is RNase E-dependent. The RNase E-independent 5′ ends (x) could correspond to an RNase III cleavage that was previously mapped *in vivo* (29). The same experiment was performed with the Δ*rhlB* strain, with or without IPTG (Fig. 2, lane 5 and 6, respectively). The RNase E-dependent 5′ ends are detectable in Δ*rhlB* (lane 5), but the signal is weaker than in wild type (lane 2) and close to the background control (lane 6). In contrast, the 5′ end of the primary transcript (+1) is more

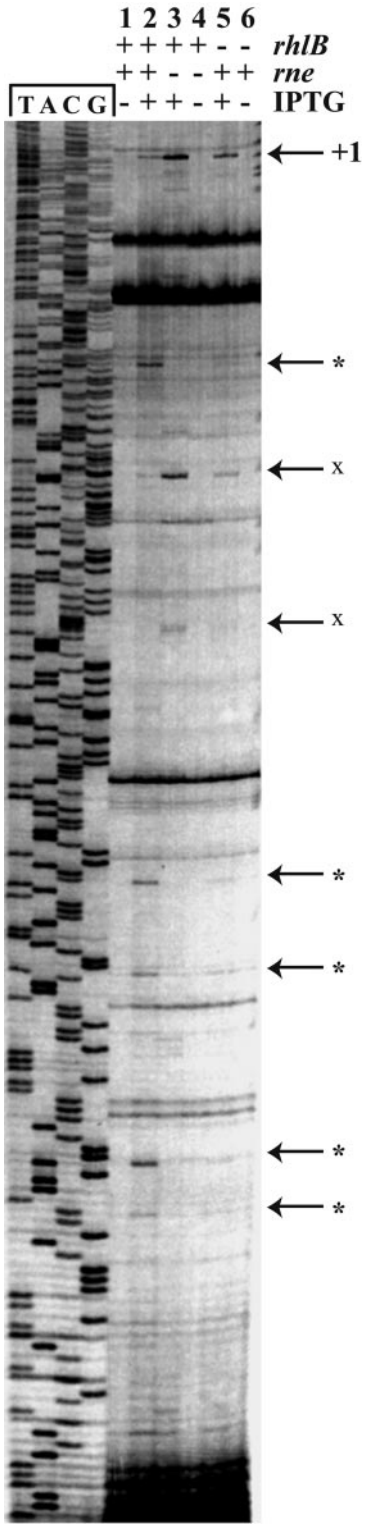

Fig. 2. Analysis of the 5′ end of the T7-*lacZ* message by primer extension. Lanes 1, 4, and 6 show, respectively, wild type, *rne1*, and Δ*rhlB* without IPTG; lanes 2, 3, and 5 are the same but with IPTG. The *lacZ* DNA sequence, run in parallel, is shown to the left. Signals corresponding to mRNA fragments whose production is RNase E dependent or independent are marked by * or x, respectively. From bottom to top, the sequences of the RNase E cleavage sites are as follows: gaau/ccga (+398), acgg/agaa (+393), acgg/ucaa (+369), and gacc/uauc (+356). The fifth site could not be precisely located. The coordinates in parentheses indicate the 5′ end of the cleavage product (underlined). The numbering takes +1 as the 5′ nucleotide of the T7-*lacZ* message (5′-ggaauu . . .).

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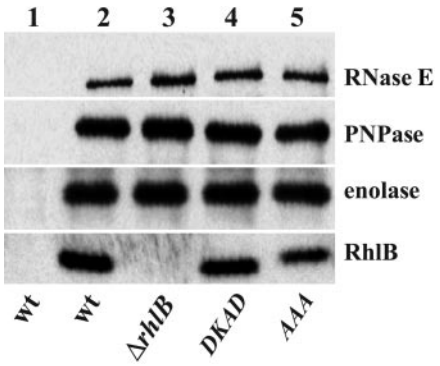

Fig. 3. Immunoprecipitation using antibodies raised against RNase E. Extracts from wild-type (lane 2), Δ*rhlB* (lane 3), $rhlB^{DKAD}$ (lane 4), and $rhlB^{AAA}$ (lane 5) strains were analyzed by Western blotting with the antibodies against the proteins noted to the right. Lane 1 is a control in which the wild-type extract was immunoprecipitated by using preimmune serum.

abundant in the Δ*rhlB* strain (lane 5) than in wild type (lane 2). These results show that RNase E cleavages in the 5′ end of the T7-*lacZ* mRNA are inhibited in the Δ*rhlB* strain. The decreased RNase E cleavage in the 5′ end of the T7-*lacZ* mRNA correlates with the increase in functional full-length T7-*lacZ* mRNA (Table 1 and Fig. 1), suggesting that these cleavages are involved in the inactivation of the T7-*lacZ* mRNA.

The Enzymatic Activity of RhlB Is Required for Its Role in T7-*lacZ* mRNA Degradation. If the absence of RhlB affects RNase E activity, this effect could be due to either allosteric control of RNase E activity by the binding of RhlB or a direct role for the enzymatic activity of RhlB. In the former case, the enzymatic activity of RhlB would not be necessary. In the conserved sequences of the DEAD-box proteins, the DEAD motif is involved in ATP hydrolysis and the SAT motif is involved in coupling ATPase and helicase activity (18). We constructed two mutants, $RhlB^{DEAD\rightarrow DKAD}$ and $RhlB^{SAT\rightarrow AAA}$. These mutants were introduced into the ENS134 strain by replacing the wild-type *rhlB* allele. We have shown previously that the $RhlB^{DKAD}$ is inactive in an *in vitro* ATPase assay (6). Far Western blots showed that the RhlB mutants bind to RNase E *in vitro* (data not shown). Their interaction *in vivo* was tested by immunoprecipitation with RNase E antibody. The degradosome components in the immunoprecipitates were analyzed by Western blotting (Fig. 3). Fig. 3, lane 1 is a control with preimmune serum. Lanes 2–5 show the results with wild type, Δ*rhlB*, $rhlB^{DKAD}$, and $rhlB^{AAA}$. The mutant RhlB proteins are present as components of the RNA degradosome (lanes 4 and 5). In all of these strains, we detected enolase and PNPase, showing that the absence of RhlB or the presence of the mutants did not interfere with the association of the other degradosomal proteins. The relative levels of β-galactosidase are 4.1- and 4.0-fold for $rhlB^{DKAD}$ and $rhlB^{AAA}$, respectively (Table 1). Thus, the extent of overproduction of β-galactosidase with these mutants is the same as in the Δ*rhlB* strain. These results show that the effect on the T7-*lacZ* mRNA of point mutations that inactivate RhlB is the same as the deletion of the gene. We conclude that the enzymatic activity of RhlB is required for its role in T7-*lacZ* mRNA degradation.

Deletion of the *rhlB* Gene Does Not Generally Inhibit RNase E Activity. The decreased RNase E cleavage of the T7-*lacZ* mRNA could be due to a global inhibition of RNase E activity in the Δ*rhlB* strain. We recently showed that small mRNA decay fragments accumulate in Δ*rhlB* and $pcnB^-$ strains and that accumulation was the strongest in a Δ*rhlB* $pcnB^-$ double mutant (12). The *pcnB* gene encodes poly(A) polymerase, which is involved in the exonucleolytic degradation of mRNA fragments. The accumulation of intermediates in mRNA decay could interfere with RNase E activity by competing with full-length mRNA. We tested this possibility by using an *rne-lacZ* fusion that is an indicator of RNase E activity in the cell. Briefly, the message that encodes RNase E is exceptionally sensitive to inactivation by RNase E due to elements in its 5′ UTR. This sensitivity permits the self-regulation of RNase E expression (30, 31). Previous work has shown that β-galactosidase expression from the *ez1* gene, which is a fusion between the 5′ regulatory elements of the *rne* gene and the coding sequence of *lacZ*, makes *lacZ* expression sensitive to the level of RNase E activity in the cell (24). The *ez6* fusion, in which the regulatory elements in the 5′ UTR have been disrupted, is a control for effects on transcription or plasmid copy number. In Table 3, β-galactosidase expression was measured in strains harboring *ez1* or *ez6*. The differences between the β-galactosidase levels of wild type harboring the *ez6* control and the mutant strains are small and not significant. Derepression was calculated as the ratio of β-galactosidase activity of *ez1* to *ez6* normalized to this ratio in wild type. The 2.6-fold derepression in the *rne131* strain (AC27) agrees well with previous measurements by Western blotting showing that the mutant RNase E protein is overexpressed 2.8-fold (23). In the Δ*rhlB* and $pcnB^-$ strains, there is no detectable derepression. In agreement with this result, we were unable to detect differences by Western blotting (data not shown). However, the 1.7-fold derepression in the Δ*rhlB* $pcnB^-$ strain suggests that the very high levels of mRNA decay intermediates in this strain could interfere with RNase E activity. Because Δ*rhlB* by itself does not derepress expression of the *ez1* fusion, it seems unlikely that the stabilization of the T7-*lacZ* message can be explained by general inhibition of RNase E activity. This conclusion is consistent with previous characterizations of Δ*rhlB* showing that growth is not impaired and that there is no obvious defect in the RNase E-mediated maturation of tRNA and rRNA, or in the degradation of mRNAs synthesized by *E. coli* RNA polymerase (V.K., unpublished results, and refs. 12 and 32).

Table 3. β-Galactosidase activity

Strain	Genotype	Activity (±SD, %) *ez1*	Activity (±SD, %) *ez6*	*ez1*/*ez6* Derepression
MC1061	Wild type	1,220 (±6.3)	1,660 (±8.6)	1.0
AC27	*rne131*	3,320 (±8.7)	1,730 (±2.3)	2.6
SVK1	Δ*rhlB*	1,470 (±4.9)	1,940 (±9.9)	1.0
SVK45	$pcnB^-$	1,050 (±5.6)	1,480 (±4.7)	1.0
SVK46	Δ*rhlB* $pcnB^-$	1,970 (±4.4)	1,580 (±5.3)	1.7

The results are the average of at least three independent measurements. Activities were measured as described (23). The SD as a percentage is in parentheses. Derepression is the ratio of the activity with the *ez1* fusion to the activity with the *ez6* fusion normalized to this ratio in wild type.

Deletion of the *rhlB* Gene Stabilizes Other T7 Messages. To test whether the effect of the Δ*rhlB* mutant is specific to the *lacZ* transcript or due to transcription by the T7 RNA polymerase, we examined the expression of two recombinant mouse proteins, krox20 and engrailed, expressed from plasmids in which the gene is under the control of a T7 promoter. Previous work showed that the expression of these recombinant proteins is significantly improved by the *rne131* mutation (22). A third protein, the HTLV protease, was also tested, but we were unable to detect a specific signal by Western blotting, apparently due to loss of activity of the antiserum. Plasmids harboring the genes encoding the recombinant proteins were transformed into ENS134 and into the isogenic SVK4 (Δ*rhlB*) and ENS134-2 (*rne131*) strains. Expression was induced by IPTG and analyzed by Western

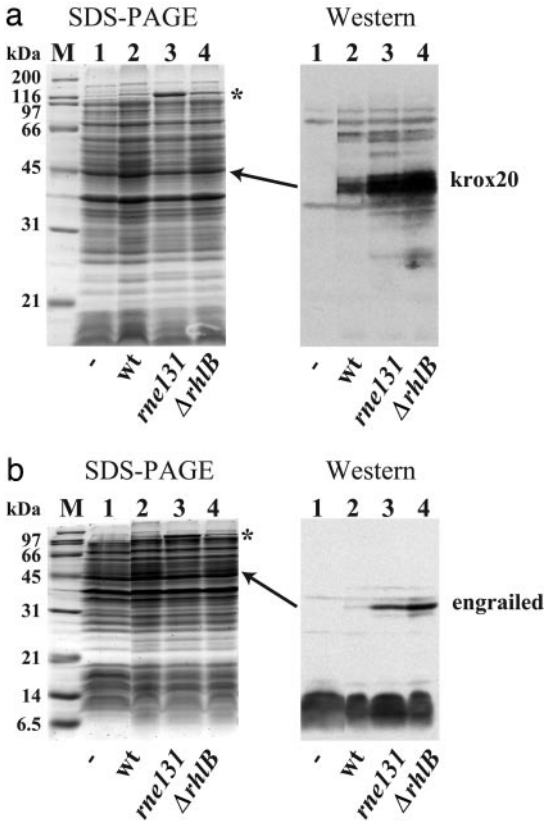

Fig. 4. SDS/PAGE and Western blots from *E. coli* strains expressing recombinant krox20 (*a*) or engrailed (*b*) transcribed by T7 RNA polymerase. In each panel, lanes 1–4 are, respectively, as follows: BL21(DE3)/pET11a (−), ENS134/krox20 or engrailed (wt), ENS134-2/krox20 or engrailed (*rne131*), SVK4/krox20 or engrailed (Δ*rhlB*). In *a*, 12% SDS gel was stained with Sypro Orange and visualized by fluorimaging. In *b*, same as *a*, except that the proteins were separated on a 15% gel. The Western blots were developed by enhanced chemiluminescence (ECL) detection. The krox20 and engrailed proteins are not visible on the SYPRO Orange-stained SDS/PAGE. Their approximate migration, based on the Western blots, is indicated by the arrow. The asterisk to the right of the SDS/PAGE indicates the position of the β-galactosidase expressed from the chromosomal T7-*lacZ* gene.

blotting (Fig. 4). The total protein loaded in each lane is equivalent (SDS/PAGE). In each panel of Fig. 4, lane 1 is a background control showing the signal from BL21(DE3) transformed with pET11a. BL21(DE3) is the isogenic parent of ENS134. As expected, the expression in the *rne131* mutant (lane 3) is significantly higher than in the wild-type control (lane 2). The expression also increases significantly in the Δ*rhlB* strain (lane 4). The effect of Δ*rhlB* is equal to or slightly stronger than the *rne131* mutation, suggesting an important role for RhlB in the inactivation of these messages. The β-galactosidase expression from the chromosomal T7-*lacZ* gene is visible in the SDS/PAGE (asterisk, clearly resolved on the 12% gel used to separate krox20). Consistent with the enzyme assays showing ≈3-fold more β-galactosidase activity in *rne131* than in Δ*rhlB* (Table 1), β-galactosidase expression in *rne131* (lane 3) is higher than in Δ*rhlB* (lane 4). Thus, in contrast to the RNase E-dependent inactivation of the T7-*lacZ* mRNA, which is partially RhlB-dependent, the RNase E-dependent inactivation of the krox20 and engrailed messages is fully RhlB-dependent.

Discussion

The principal result of this work is that the DEAD-box RNA helicase, RhlB, facilitates the inactivation by RNase E of messages synthesized by bacteriophage T7 RNA polymerase. This conclusion is supported by the primer extension analysis (Fig. 2), which shows that the Δ*rhlB* mutation impairs RNase E cleavages in the 5′ end of the T7-*lacZ* mRNA. This decrease in RNase E cleavage correlates with an increase in full-length *lacZ* mRNA (Fig. 1) and an increase in the level of β-galactosidase activity (Table 1). These results provide evidence for a functional interaction between RhlB and RNase E. Furthermore, until now, the RNA helicases identified in mRNA degradation have been characterized as part of exonucleolytic degradation pathways involving enzymes such as the prokaryotic PNPase or the eukaryotic exosome (13, 14). Our analysis of the self-regulation of RNase E expression in the Δ*rhlB* strain (Table 3), which is normal, argues against a general inhibition of RNase E activity. The strains used in Table 3 are the same strains in which we previously demonstrated the accumulation of structured mRNA decay intermediates due to the disruption of the RhlB-PNPase pathway (12). Thus, an inhibition of RNase E activity by the mRNA decay intermediates that accumulate in the Δ*rhlB* strain is unlikely. The lack of a general effect on RNase E is consistent with recent work suggesting that the Δ*rhlB* mutation has at most a small effect on the lifetime of messages synthesized by *E. coli* RNA polymerase (12, 32). Furthermore, the results in the Northern blot (Fig. 1) suggest that the intercistronic RNase E cleavage between *lacZ* and *lacY′* is not affected because the abundance of the processed 3.2-kb species increases in the Δ*rhlB* strain. These results suggest that RhlB is not generally required for RNase E activity *in vivo*. It should be noted that this is similar to the RhlB-PNPase pathway because RhlB is not required for PNPase activity except under particular circumstances involving the degradation of highly structured mRNA decay intermediates.

The increased expression of krox20 and engrailed (Fig. 4) suggests that the effect of Δ*rhlB* is general for messages synthesized by T7 RNA polymerase. Previous work has shown that the effect of the *rne131* mutation, in which the entire noncatalytic region of RNase E is disrupted, on the T7-*lacZ* message is equivalent to the effect of an *rne* thermosensitive mutation, in which the endoribonuclease activity is abolished at the nonpermissive temperature (21, 22). Thus, the exceptional sensitivity of the T7-*lacZ* message to RNase E is mediated by elements in the noncatalytic region (22, 23). The effect of Δ*rhlB* on the expression of krox20 and engrailed is comparable with the effect of *rne131* whereas its effect on the T7-*lacZ* message is weaker than *rne131*. Regardless of this difference, these results show that RhlB is important in the inactivation and degradation of the T7 messages. There are several differences between T7-*lacZ*, and krox20 and engrailed that could explain the differential effect of Δ*rhlB*, including the length and sequence of the messages, the signals for translation (*lac* vs. T7), and the number of copies of the gene. It is unlikely that the effect of Δ*rhlB* on krox20 and engrailed is due to a trivial effect on plasmid copy number because the Δ*rhlB* mutation does not affect pBR322 replication (V.K., unpublished results). The stringent requirement for RhlB in the inactivation of krox20 and engrailed could, for example, be due to high-level transcription from multicopy genes or differences in the translation signals.

The distinctive features of T7 transcription are the synthesis of long stretches of ribosome-free mRNA due to rapid elongation and the relative insensitivity of the RNA polymerase to transcription termination by Rho, a factor that inhibits the synthesis of untranslated mRNA by *E. coli* RNA polymerase (33). If the RhlB-RNase E pathway in *E. coli* were involved in the degradation of ribosome-free mRNA, this function would be complementary to transcription termination by Rho. Messages that are under the control of a translational repressor are a source of ribosome-free mRNA that might escape transcription termination by Rho. Indeed, it has already been shown that the repressed threonine synthetase mRNA is exceptionally sensitive to degra-

dation by RNase E and that this sensitivity is abolished by the *rne131* mutation (34). That is, full-length translationally repressed messages accumulate in the *rne131* strain whereas in wild type they are rapidly degraded. Coupling mRNA degradation to translational control makes repression irreversible. The RhlB-RNase E degradation pathway could also be involved in the posttranscriptional regulation of gene expression by small antisense RNAs such as RhyB because RhyB-mediated mRNA degradation, which is RNase E-dependent, is slower in the *rne131* strain (35, 36). In contrast to the situation in uninfected *E. coli*, bacteriophage T7 messages are unusually long-lived (20-min half-life), and it has been suggested that this stability is due to inhibition of the host mRNA degradation machinery during an infection (37). Bacteriophage T7 synthesizes a protein kinase that phosphorylates numerous host proteins. Expression of this kinase in the ENS134 strain leads to the phosphorylation of RNase E and RhlB, and the stabilization of the T7-*lacZ* message (38). Thus, messages synthesized by the T7 RNA polymerase during an infection might be protected from inactivation by the RhlB-RNase E pathway due to phosphorylation of these enzymes by the protein kinase.

Our results show that point mutations inactivating RhlB have the same effect as deleting the *rhlB* gene (Table 1). Furthermore, the inactive RhlB protein is associated with RNase E as a component of the RNA degradosome (Fig. 3). Thus, it is unlikely that the physical interaction between RhlB and RNase E is involved in an allosteric regulation of RNase activity. The requirement for enzymatic activity suggests a role for RNA unwinding or remodeling. Because RNase E is single-strand specific, RhlB could counteract the collapse of RNase E cleavage sites into double-stranded structures. Experiments *in vitro* using the purified RNA degradosome show no effect of ATP on the maturation by RNase E of 5S rRNA from its 9S precursor (A.J.C. and L.P., unpublished results). This result is not unexpected because the RNase E sites in the 9S precursor are in single-stranded regions that are unlikely to collapse into double-stranded structures (39). Similarly, we have been unable to detect an effect of ATP on the cleavage of an mRNA fragment from the 5′ end of *lacZ*. However, these experiments need to be extended to an analysis of the full-length *lacZ* message as well as the krox20 and engrailed messages. Another possibility is that RNA binding proteins are involved. Ribosome-free mRNA is unlikely to be protein-free *in vivo*. Because NPH-II, a DExD/H protein from vaccinia virus, has been shown to displace RNA binding proteins (40), RhlB could be involved in the disruption or remodeling of ribonucleoprotein to facilitate RNase E cleavage. It should be interesting in future experiments to elucidate the mechanism by which RhlB facilitates the degradation of ribosome-free mRNA.

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