Differential Control of the Rate of 5'-End-Dependent mRNA Degradation in *Escherichia coli*

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Many *Escherichia coli* mRNAs are degraded by a 5'-end-dependent mechanism in which RppH-catalyzed conversion of the 5'-terminal triphosphate to a monophosphate triggers rapid endonucleolytic cleavage by RNase E. However, little is understood about what governs the decay rates of these transcripts. We investigated the decay of three such messages—*rpsT* P1, *yfcZ*, and *ydfG*—to characterize the rate-determining step in their degradation. The steady-state ratio of monophosphorylated to triphosphorylated *rpsT* P1 and *yfcZ* mRNA indicates that their decay rate is limited by cleavage of the monophosphorylated intermediate, making RNase E critical for their rapid turnover. Conversely, the decay rate of *ydfG* is limited by generation of the monophosphorylated intermediate; therefore, either RNase E or its less abundant paralog RNase G is sufficient for rapid *ydfG* degradation. Although all three transcripts are stabilized when RppH is absent, overproducing RppH does not accelerate their decay, nor does RppH overproduction appear to influence the longevity of most other messages that it targets. The failure of excess RppH to hasten *rpsT* P1 and *yfcZ* degradation despite increasing the percentage of each that is monophosphorylated is consistent with the observation that pyrophosphate removal is not the rate-limiting step in their decay. In contrast, neither the *ydfG* decay rate nor the fraction of *ydfG* transcripts that are monophosphorylated increases when the cellular concentration of RppH is raised, suggesting that, for some RppH targets, the rate of formation of the monophosphorylated intermediate is limited by an ancillary factor or by a step that precedes pyrophosphate removal.

An important mechanism for controlling gene expression in all organisms is the rate at which mRNA is degraded, which enables them to modulate cellular mRNA concentrations and adapt rapidly to environmental changes. The longevity of distinct transcripts within the same cell can differ substantially. For example, although the average half-life of mRNA in *Escherichia coli* is about 6 min (6, 29), lifetimes can vary by up to 2 orders of magnitude, with commensurate effects on protein synthesis.

E. coli cells appear to lack a 5' exoribonuclease, and 3' exonucleolytic degradation is inhibited by the stem-loop structures typically present at mRNA 3' ends. Consequently, endonucleolytic cleavage is thought to be a critical early event in mRNA turnover in that organism (5). The most important endonuclease for degrading mRNA in *E. coli* is RNase E, whose inactivation stabilizes most messages (3, 24–26, 30). This enzyme has low cleavage site specificity, preferring to cut in single-stranded regions that are AU rich (23). The resulting 5' and 3' fragments are then rapidly degraded to mononucleotides by a combination of further endonucleolytic cleavage and 3' exonucleolytic digestion (5).

RNase E and its less abundant paralog RNase G can gain access to cleavage sites in mRNA either directly or by a 5'-end-dependent mechanism (5). The direct-access pathway allows cleavage of triphosphorylated primary transcripts, whereas 5'-end-dependent access requires prior conversion of the 5'-terminal triphosphate to a monophosphate by the RNA pyrophosphohydrolase RppH (11, 13). The monophosphorylated 5' end thereby generated can bind in a discrete pocket on the surface of RNase E and accelerate internal RNA cleavage by the catalytic active site of the same enzyme (10, 21). RppH targets hundreds of *E. coli* transcripts, as evidenced by their increased longevity and concentration in cells devoid of RppH activity (13). However, apart from the protective effect of a 5'-terminal stem-loop (1, 4, 8, 9, 15), which inhibits both pyrophosphate removal by RppH and 5'-monophosphate-assisted cleavage by RNase E (13, 21), little is under-

stood about the features of mRNAs that govern their susceptibility to rapid degradation by the 5'-end-dependent pathway.

To better understand the role that RppH plays in controlling mRNA lifetimes, we compared the rates of 5' pyrophosphate removal and subsequent RNase E cleavage for three representative *E. coli* transcripts that are degraded by a 5'-end-dependent mechanism. In addition, we examined whether an excess of RppH affects the concentration, phosphorylation state, and half-life of the mRNAs that it targets. Our data indicate that either pyrophosphate removal or RNase E cleavage can determine the rate of 5'-end-dependent degradation and suggest that the rate at which RppH converts the 5'-terminal triphosphate to a monophosphate may sometimes be limited by a prior event or another cellular factor.

MATERIALS AND METHODS

Strains and plasmids. Measurements of protein levels and of mRNA lifetimes and 5' phosphorylation states were performed in *E. coli* K-12 strain BW25113 and its isogenic derivatives JW2798 Δ kan, which contains an in-frame deletion of the *rppH* coding region, and JW3216 Δ kan, which contains an in-frame deletion of the *rng* coding region (2, 13). Alternatively, the measurements were performed in SK9714 (*rne* Δ 1018::bla) containing either pRne-SG1 (wild-type RNase E) or pRne-SG4 (RNase E-R169Q) (16). The effect of RNase E inactivation in the presence or

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absence of RNase G was examined in isogenic derivatives of BW25113 that contained a wild-type or temperature-sensitive *rne* allele (*rne-1*, also known as *ams-1*) (22) and an intact or deleted *rng* gene.

The chromosomal *rppH* gene of BW25113 was tagged to encode an RppH derivative (RppH-FH) bearing carboxy-terminal FLAG and hexahistidine tags (DYKDDDDKGGHHHHHH) by allelic exchange, as described previously (14) but with the following modifications. The inserted DNA segment, along with ~0.75 kb of chromosomal DNA upstream and downstream of the *rppH* termination codon, was amplified by SOE (splicing by overlapping extension)-PCR, cloned into plasmid pRE112, and introduced into BW25113 by conjugation with *E. coli* strain S17-1 λ . Single recombinants were selected on minimal morpholinepropanesulfonic acid (MOPS) agar containing glucose (0.2%) and chloramphenicol (20 μ g/ml), and double recombinants were then selected on LB agar containing 5% sucrose but no sodium chloride and verified by PCR and immunoblotting.

Plasmid pPlacRppH, which encodes wild-type RppH synthesized under the control of an isopropyl- β -D-thiogalactopyranoside (IPTG)-inducible *lacUV5* promoter, has been described previously (13). Plasmid pPlacRppH-FH was constructed from pPlacRppH to add tandem FLAG and hexahistidine tags (DYKDDDDKGGHHHHHH) to the carboxyl terminus of plasmid-encoded RppH. Plasmid pOmpA Δ 104Q-F was constructed from pOmpA Δ 104Q (1) to add a FLAG tag at the carboxyl terminus of OmpA. Plasmids pYdfG1 and pYfcZ1 were constructed by cloning the *ydfG* or *yfcZ* gene between the EcoRI and PstI sites of plasmid pBR322fd, a derivative of pBR322 in which a 0.35-kb HindIII fragment of pLBU1 containing the bacteriophage fd terminator (17) had been inserted at the HindIII site to block transcription of the *bla* gene from promoters upstream of its natural promoter without impairing transcription of the *tet* gene.

Measurement of RNA lifetimes. To examine the effect of RppH on rates of mRNA degradation, *E. coli* cells were grown to mid-log phase at 37°C in MOPS medium containing glucose (0.2%) and IPTG (10 μ M, which induced RppH overproduction in cells containing pPlacRppH), and total cellular RNA was extracted at time intervals after inhibiting transcription with rifampin (0.2 mg/ml). To determine the effect of an RNase E 5' sensor defect on mRNA decay, the cells were instead grown in LB medium. To examine the consequences of RNase E inactivation in either the presence or absence of RNase G, cells that contained a wild-type or temperature-sensitive *rne* allele and had been growing exponentially at 30°C in MOPS-glucose medium were shifted to 44°C 10 min before rifampin addition. For quantitative reverse transcription-PCR (qRT-PCR) analysis, cDNA was synthesized from 1 μ g of DNase I-treated (Ambion) total RNA by using SuperScript III (Invitrogen), along with 0.2 μ g of random-hexamer primers (Qiagen), according to the manufacturer's instructions. qPCR was carried out in triplicate in a volume of 25 μ l, which included 12.5 μ l of SYBR green supermix (Roche). After heating for 10 min at 95°C, the qPCR mixtures were subjected to 40 amplification cycles (15 s at 95°C, 1 min at 55°C). The initial concentration of each cDNA was determined by fitting the data to a two-parameter MAK2 model (7, 27), using an offset of four cycles after the second derivative maximum to approximate the first derivative maximum, and then normalized to that of 16S rRNA. mRNA half-lives were calculated by linear regression analysis of a semilogarithmic plot of mRNA concentration versus time after rifampin addition.

For Northern blot analysis, equal amounts of total RNA (10 μ g) from cells that contained plasmid pYdfG1 or pYfcZ1 and had been treated with rifampin for various periods of time were subjected to gel electrophoresis on either polyacrylamide (4% to 6%) containing 8 M urea or 1.0% agarose containing 2.4% formaldehyde. RNA was transferred to a Hybond-XL membrane (GE Healthcare) by electroblotting (polyacrylamide gels) or overnight capillary transfer (agarose gels). The blots were UV cross-linked and probed with a 5'-radiolabeled oligonucleotide complementary to either *rpsT*, *ydfG*, or *yfcZ* mRNA (see Table S1 in the supplemental material). Radioactive bands were visualized with a Storm 820 Phosphor-

Imager (Molecular Dynamics), and the band intensities were quantified by using ImageQuant software (Molecular Dynamics). mRNA half-lives were calculated by linear regression analysis.

Kinetic modeling of RppH-dependent mRNA degradation via a two-step mechanism (triP $\rightarrow$ monoP $\rightarrow$ decay products) (see below for definitions of terms) was accomplished by numerical integration. The only variables were k_{pp} and k_E , whose values were constrained by the requirement that their ratio equal the observed steady-state ratio of monophosphorylated to triphosphorylated mRNA. These two rate constants include not only the hydrolytic events themselves, but also any preliminary steps in the formation or breakdown of the monophosphorylated decay intermediate, such as steps that may precede the binding of triphosphorylated mRNA to RppH (components of k_{pp}) and the dissociation of the monophosphorylated intermediate from RppH (components of k_E). Theoretical half-lives were calculated by linear regression analysis of the mRNA remaining (triP plus monoP) at 7 to 8 evenly spaced time intervals from the moment of transcription inhibition until ~90% of the mRNA had been degraded.

5'-end mapping. The 5' ends of *ydfG* and *yfcZ* mRNA were mapped by rapid amplification of cDNA ends (RACE). To convert all 5' ends to ligatable monophosphates, 5 μ g of total *E. coli* RNA was treated with 2 units of tobacco acid pyrophosphatase (TAP) (Epicentre Biotechnologies) for 2 h at 37°C. Following phenol-chloroform extraction and ethanol precipitation, 2.5 μ g of the TAP-treated RNA was ligated to 1.25 μ g of an RNA oligonucleotide (RLM-RNA) (see Table S1 in the supplemental material) with T4 RNA ligase (Epicentre Biotechnologies), according to the manufacturer's instructions. The RNA was converted to cDNA by using SuperScript III (Invitrogen) and either *ydfG* rev or *yfcZ* rev as a primer (see Table S1 in the supplemental material). The cDNA was then PCR amplified by using either RACE+ or RACEnest as a forward primer and *ydfG* rev or *yfcZ* rev as a reverse primer, and the PCR products were gel purified and sequenced to identify the mRNA 5' end.

PABLO analysis. To examine the effect of RppH on the 5' phosphorylation state of mRNA, total cellular RNA was extracted from *E. coli* cells that had been grown to mid-log phase at 37°C in MOPS medium containing glucose (0.2%) and IPTG (10 μ M, which induced RppH overproduction in cells containing pPlacRppH). The phosphorylation states of individual *E. coli* transcripts were determined by PABLO analysis, i.e., splinted ligation of monophosphorylated RNA to a 32-nucleotide (nt) DNA oligonucleotide (X32) in the presence of a bridging DNA oligonucleotide specific for a single RNA 5' end (*Y_{rpsT}*, *Y_{yfcZ}*, or *Y_{ydfG}*), as previously described (12) (see Table S1 in the supplemental material). Detection of *yfcZ* and *ydfG* mRNA was enhanced by examining total RNA extracted from cells that contained plasmid pYfcZ1 or pYdfG1, respectively. To improve the electrophoretic separation of the ligated and unligated reaction products, 400 pmol of a transcript-specific 10-23 DNase (28) was included in each PABLO reaction to generate a shorter 5'-terminal fragment by site-specific cleavage within codon 12 (*rpsT*) or 21 (*yfcZ* and *ydfG*) (see Table S1 in the supplemental material). The reaction products were detected by Northern blot analysis with transcript-specific probes. To calculate the percentage of each transcript that was monophosphorylated, PABLO ligation yields were divided by the ligation yield measured for the same transcript after treating total RNA with 2 units of TAP for 2 h at 37°C to convert all 5' ends to monophosphates. All of the PABLO measurements were performed three or more times, and a mean and standard deviation were calculated.

Microarray analysis. Total cellular RNA was extracted in triplicate from cultures of either BW25113 or JW2798 Δ *kan* growing exponentially at 37°C in MOPS medium containing glucose (0.2%). cDNA was prepared by random-hexamer-primed reverse transcription with SuperScript II (Invitrogen), fragmented with DNase I (GE Biosciences), biotin labeled with the GeneChip DNA-labeling reagent (Affymetrix) and terminal deoxynucleotidyl transferase (Promega), and used to probe *E. coli* Genome 2.0 arrays (Affymetrix). The microarrays were scanned with an Affymetrix GeneChip Scanner 3000, and the raw data were scaled and

FIG 1 RppH overproduction in *E. coli*. (A) Relative concentration of RppH in wild-type and overproducing cells. Protein extracts from cells that contained either a single chromosomal copy of the epitope-tagged *rppH* gene (RppH-FH) or, in addition, a multicopy plasmid encoding epitope-tagged RppH were serially diluted with a protein extract from cells that lacked RppH-FH, and their RppH-FH content was analyzed by immunoblotting. (B) Solubility of RppH overproduced in *E. coli*. A protein extract from cells that overproduced untagged RppH from a multicopy plasmid (T) was separated by centrifugation into soluble (S) and insoluble (P) fractions, and the fractions were examined by immunoblot analysis with polyclonal anti-RppH antibodies. As controls, the immunoblots were also probed for the cytoplasmic protein DnaK (soluble) or a FLAG-tagged derivative of the outer membrane protein OmpA (insoluble).

quantified with Affymetrix GCOS software. Triplicate values were averaged, and significant probesets were calculated by *t* test. A *P* value of < 0.01 was used as a cutoff for statistical significance.

Subcellular fractionation and immunoblotting. Subcellular fractionation of *E. coli* extracts was performed as described previously (32). For immunoblot analysis, cellular proteins were separated by SDS-PAGE (15% polyacrylamide) and transferred to a Hybond-P membrane (GE Healthcare) by electroblotting. Following incubation with either a polyclonal antiserum or a monoclonal antibody, and then with goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad), protein bands were visualized by enhanced chemiluminescence (Bio-Rad). Serial dilution of cellular protein prior to immunoblot analysis was achieved by diluting cell extracts that contained RppH with a cell extract that did not contain RppH to ensure that equal amounts of total protein were loaded in each lane.

Polyclonal rabbit anti-RppH antibodies (Covance; raised against purified RppH bearing an amino-terminal hexahistidine tag) and monoclonal anti-DnaK (Assay Designs), anti-FLAG (Sigma), and anti-His (Clontech) antibodies were used at dilutions of 1:1,000, 1:2,000, 1:5,000, and 1:5,000, respectively.

RESULTS AND DISCUSSION

Effect of RppH overproduction on mRNA concentrations. Previously, we screened for RppH targets in *E. coli* by using microarray analysis to compare transcript levels in cells that lacked the chromosomal *rppH* gene and instead contained a multicopy plasmid encoding either wild-type RppH or a catalytically inactive RppH mutant (RppH-E53A) under the control of a heterologous promoter and 5' untranslated region (13). In this manner, we identified hundreds of messages whose concentrations were significantly elevated in cells lacking RppH activity. However, those experiments may have exaggerated the influence of RppH in *E. coli* if its cellular concentration was unnaturally high due to production from a multicopy plasmid.

To ascertain by how much RppH had been overproduced in those experiments, we compared its concentration in cells that

FIG 2 Effect of RppH overproduction on the concentration of its targets. *E. coli* mRNAs downregulated by RppH were identified by using microarray analysis to compare the concentration in cells that either contained or lacked the chromosomal *rppH* gene ($\Delta rppH$ /WT) or that lacked the chromosomal *rppH* gene and instead contained a multicopy plasmid encoding wild-type RppH or catalytically inactive RppH-E53A (Excess E53A/Excess WT) (13). The effect of RppH, measured in triplicate at either a normal or an elevated protein concentration, was calculated for 462 mRNAs and graphed, using a *P* value of < 0.01 as a threshold for significance, and the best-fit line was calculated by linear regression (slope = 0.88). Messages whose half-lives were subsequently compared in wild-type and RppH-overproducing cells are represented by white (*rpsT*, *yfcZ*, and *ydfG*) or gray (*csrB*, *hisD*, and *malP*) data points.

contained either a single chromosomal copy of the *rppH* gene or the multicopy *rppH* plasmid. To facilitate detection, both copies of the gene were carboxy-terminally tagged with a FLAG epitope and six histidine residues. The relative abundance of RppH was then estimated by immunoblot analysis of unfractionated cell extracts (Fig. 1A). These experiments showed that the concentration of RppH in *E. coli* increases ~500-fold when overproduced from the multicopy *rppH* plasmid. Furthermore, about half of the untagged RppH in cells containing the original multicopy plasmid was soluble and therefore potentially functional, as judged by separating a cell extract into soluble and insoluble fractions (Fig. 1B).

The realization that RppH was present at quite a high concentration in the prior microarray study prompted us to conduct a new microarray experiment in which transcript levels were compared in *E. coli* cells that contained wild-type RppH at its normal concentration (plasmid-free cells bearing a single, untagged chromosomal copy of the *rppH* gene) or no RppH at all (plasmid-free cells from which the *rppH* gene had been deleted). Despite the large difference in RppH concentrations, the RppH-sensitive messages identified in this manner were almost identical to those identified previously, as was the magnitude of the effect of RppH on their abundance (Fig. 2; see Table S2 in the supplemental material). These findings indicate that neither the number of *E. coli* mRNAs targeted by RppH nor the degree to which they are downregulated is significantly affected by RppH overproduction, raising the possibility that their decay rates are not limited by the cellular concentration of RppH.

Effect of RppH overproduction on mRNA lifetimes. To test directly whether the degradation of these messages is insensitive to increasing the concentration of RppH, we compared the half-lives of three monocistronic RppH targets in isogenic *E. coli* strains that contained either a normal amount of RppH (untagged), an ele-

FIG 3 Effect of RppH overproduction on mRNA decay rates, as determined by quantitative RT-PCR. Total RNA was extracted at time intervals after rifampin addition to wild-type, $\Delta rppH$, and RppH-overproducing *E. coli* cells, and equal amounts were analyzed by qRT-PCR with primers specific for the *rpsT* P1, *yfcZ*, or *ydfG* transcript or for 16S rRNA (a stable internal standard). The mRNA levels in each sample were calculated by MAK2 analysis (7, 27), normalized to the concentration of 16S rRNA, and plotted as a function of time. Each point is the average of three measurements.

vated concentration of RppH (untagged), or no RppH whatsoever. The cells were grown to mid-log phase, rifampin was added to block transcription initiation, and total cellular RNA was extracted at time intervals and analyzed by qRT-PCR with primers specific for the *rpsT* P1 transcript, which encodes ribosomal protein S20; *ydfG* mRNA, which encodes an NADP⁺-dependent 3-hydroxy acid dehydrogenase; *yfcZ* mRNA, which encodes a conserved protein of unknown function; and 16S rRNA (a stable internal standard). As expected, the half-lives of all three mRNAs were substantially (~5-fold) longer in cells lacking RppH (Fig. 3; see Table S3 in the supplemental material). However, a large increase in the cellular concentration of RppH had almost no effect on the decay rates of these transcripts.

To corroborate these observations, we measured the half-lives of the same three messages in wild-type and RppH-overproducing cells by Northern blot analysis (Fig. 4A). In each case, the calculated rate of decay was similar to that measured by quantitative

FIG 4 Effect of RppH overproduction on mRNA decay rates, as determined by Northern blotting. (A) Northern blot analysis of the decay of *rpsT*, *yfcZ*, and *ydfG* mRNA. *E. coli* cells containing normal or elevated levels of RppH were transformed with a multicopy plasmid bearing the *ydfG* or *yfcZ* gene, total RNA was extracted at time intervals after rifampin addition, and equal amounts were analyzed by Northern blotting with probes complementary to the coding region of *yfcZ* or *ydfG* mRNA or to a region of the *rpsT* 5' UTR that allowed detection of mRNA transcribed from either the P1 promoter or a downstream promoter (P2). (B) mRNA half-lives in cells containing normal or elevated levels of RppH, as determined by quantitative RT-PCR or Northern blotting. The error bars represent standard deviations.

RT-PCR (Fig. 4B; see Table S3 in the supplemental material), confirming that a substantial increase in the concentration of RppH does not significantly accelerate their degradation by the 5'-end-dependent pathway.

In addition, we tested three transcripts (*csrB*, *hisD*, and *malP*) for which the two microarray studies had come to very different conclusions about the degree to which RppH influences their abundance (Fig. 2). For none of them did increasing the concentration of RppH diminish transcript stability, as measured by quantitative RT-PCR (see Table S4 in the supplemental material). By extrapolation, it seems likely that the decay of most of the other outliers in Fig. 2 is not significantly accelerated by RppH overproduction. Neither does its overproduction at this level impede cell growth (data not shown).

Effect of RppH overproduction on 5' phosphorylation states. In principle, the failure of an elevated RppH concentration to accelerate 5'-end-dependent degradation could be explained if the rate of this process is limited, not by pyrophosphate removal, but by subsequent RNase E cleavage. The relative rates of these two steps can be determined from the steady-state ratio of monophosphorylated to triphosphorylated RNA, which should equal the ratio of the rate constants for the formation and breakdown of the monophosphorylated decay intermediate, including any preliminary or intermediate steps in these events: $\text{monoP}/\text{triP} = k_{\text{PP}}/k_{\text{E}}$, where monoP is the steady-state concentration of the full-length monophosphorylated intermediate, triP is the steady-state con-

centration of its triphosphorylated precursor, and k_{pp} and k_E are the rate constants for pyrophosphate removal and subsequent RNase E cleavage, respectively. Consequently, this ratio can be used to ascertain which of these two steps governs the rate of 5'-end-dependent mRNA degradation.

To examine the phosphorylation state of the *rpsT* P1, *yfcZ*, and *ydfG* transcripts, their 5' termini were first identified by RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE). The ratio of monophosphorylated to triphosphorylated 5' ends was then determined for each of these transcripts by PABLO, a splinted ligation assay for detecting and quantifying 5' termini that are monophosphorylated (12). This assay takes advantage of the ability of T4 DNA ligase to covalently join a DNA oligonucleotide to the 5' end of a monophosphorylated RNA, but not a triphosphorylated RNA, when the two are juxtaposed by simultaneous base pairing to a bridging DNA oligonucleotide. The ligation product and its unligated progenitor are then separated by gel electrophoresis and quantified by Northern blotting. As a normalization control, the same RNAs are analyzed by PABLO after treatment with TAP to convert their 5' ends entirely to ligatable monophosphates. The percentage of 5' ends that are monophosphorylated in *E. coli* can then be calculated from the ratio of the ligation yields before and after TAP treatment.

In wild-type cells, the fraction of transcripts that were monophosphorylated differed significantly among the three mRNAs, ranging from 17% to 60% (Fig. 5; see Table S5 in the supplemental material). Predictably, this value fell almost to zero in the absence of RppH. For *ydfG*, the ratio of monophosphorylated to triphosphorylated full-length transcripts was 1/5 (17%/83%), indicating that pyrophosphate removal from the primary transcript is much slower than RNase E cleavage of the monophosphorylated intermediate, i.e., that the decay rate of *ydfG* mRNA is controlled primarily by the rate at which the triphosphorylated transcript is converted to its monophosphorylated counterpart (k_{pp}). In contrast, the ratio was 3/2 for the *rpsT* P1 and *yfcZ* transcripts (both 60% monophosphorylated), evidence that pyrophosphate removal from these mRNAs is ordinarily somewhat faster than subsequent RNase E cleavage and that the latter step (k_E) is principally responsible for limiting their rate of decay.

We next determined whether an excess of RppH affects the relative rates of these two steps. Although overproducing RppH did not significantly affect the half-life of *rpsT* P1 or *yfcZ* (Fig. 4B; see Table S3 in the supplemental material), it raised the percentage of those transcripts that were monophosphorylated (Fig. 5; see Table S5 in the supplemental material), presumably by increasing k_{pp} . As a result, the ratio of monophosphorylated to triphosphorylated mRNA increased 3- to 7-fold. The failure of elevated RppH levels to noticeably accelerate the decay of those transcripts is consistent with the observation in wild-type cells that the rate constant for pyrophosphate removal from the 5' end of *rpsT* P1 and *yfcZ* already exceeds that for degradation of the resulting monophosphorylated intermediate; therefore, making pyrophosphate removal even faster would not be expected to hasten decay by more than 19%, as determined by kinetic modeling. Such a meager effect would be difficult to detect.

Interestingly, although PABLO analysis suggests that the half-life of *ydfG* mRNA is normally governed by the rate of pyrophosphate removal from the triphosphorylated primary transcript, increasing the cellular concentration of RppH did not affect the percentage of full-length *ydfG* transcripts that were monophos-

FIG 5 Effect of RppH overproduction on the phosphorylation state of mRNA. (A) PABLO analysis of the 5' phosphorylation state of the *rpsT* P1, *yfcZ*, and *ydfG* transcripts. Total RNA was extracted from *E. coli* cells that contained a normal or elevated level of RppH or no RppH whatsoever, and equal amounts were subjected to PABLO analysis with oligonucleotides specific for the *rpsT* P1, *yfcZ*, or *ydfG* transcript. A TAP-treated RNA sample was analyzed in parallel so that the ligation yields of fully monophosphorylated RNA could be compared. In each case, the electrophoretic resolution of the ligated and unligated reaction products was enhanced by site-specific RNA cleavage with a 10-23 DNase (28). The residual levels of monophosphorylated *rpsT* P1, *yfcZ*, and *ydfG* mRNA in the absence of RppH ($\leq 4\%$ of the total) may result from infrequent transcription initiation with AMP instead of ATP or from another, minor RNA pyrophosphohydrolase whose activity is too low to be of consequence. (B) mRNA phosphorylation states in cells containing normal or elevated levels of RppH, as determined by PABLO. The error bars represent the standard deviations of three or more independent measurements.

phorylated (Fig. 5; see Table S5 in the supplemental material) or the rate of *ydfG* degradation (Fig. 4B; see Table S3 in the supplemental material). Nevertheless, both decreased markedly when RppH was absent. These observations indicate that something other than RppH ordinarily limits the rate of production of the monophosphorylated *ydfG* intermediate and, therefore, the overall rate of *ydfG* mRNA decay. For example, efficient pyrophosphate removal from triphosphorylated *ydfG* may require not only RppH but also an ancillary protein whose concentration is limiting. Alternatively, conversion of the 5' triphosphate of *ydfG* to a monophosphate may be a two-step process in which pyrophosphate removal by RppH is triggered by a prior, RppH-independent event that is rate determining. In principle, such a triggering event might involve a change in the conformation of the *ydfG* transcript or in its interaction with ribosomes or some other cel-

FIG 6 Roles of RNase E and RNase G in RppH-dependent mRNA decay. (A) Effect of an RNase E 5' sensor defect on the degradation of *rpsT* P1, *yfcZ*, and *ydfG* mRNA. Total RNA was extracted at time intervals after rifampin addition to *E. coli* cells containing wild-type RNase E or RNase E-R169Q and analyzed by qRT-PCR as in Fig. 3. (B) Combined effect of RNase E and RNase G on the degradation of *ydfG* mRNA. (Left) Decay of *ydfG* mRNA in *E. coli* cells containing either wild-type RNase E or a temperature-sensitive RNase E mutant (*rne-1*, also known as *ams-1*, encoding RNase E-G66S) after a temperature shift from 30°C to 44°C. (Center) Decay of *ydfG* mRNA at 37°C in *E. coli* cells containing or lacking RNase G (Δ *rng*). (Right) Decay of *ydfG* mRNA in a double mutant containing both a temperature-sensitive *rne* allele and a Δ *rng* allele after shifting the temperature to 44°C. In each case, *ydfG* mRNA levels were quantified by qRT-PCR as in Fig. 3.

lular factor so as to make the 5' terminus more accessible to RppH. In either case, increasing the concentration of RppH would not be expected to affect either the half-life of *ydfG* mRNA or the percentage of *ydfG* transcripts that are monophosphorylated, yet the absence of RppH in Δ *rppH* cells would block formation of the monophosphorylated intermediate and force degradation to proceed by a slower, 5'-end-independent mechanism.

Roles of RNase E and RNase G in degrading monophosphorylated intermediates. Finally, to determine whether RNase E degrades the monophosphorylated *rpsT* P1, *ydfG*, and *yfcZ* decay intermediates generated by RppH, we examined the decay of these three messages in cells that contained a mutant form of RNase E (RNase E-R169Q [16]) unable to recognize monophosphorylated 5' ends. As expected, the half-lives of the *rpsT* P1 and *yfcZ* transcripts increased substantially (5- to 6-fold); however, the decay rate of *ydfG* was unchanged (Fig. 6A; see Table S6 in the supplemental material). The lifetime of *ydfG* mRNA also did not increase significantly upon thermal inactivation of a temperature-sensitive form of RNase E (RNase E-G66S [22]) or in the absence of RNase G, an RNase E paralog whose specificity is similar to that of RNase E but whose concentration in *E. coli* is much lower (18–20, 31). However, when RNase E was inactivated in cells lacking RNase G, the lifetime of *ydfG* mRNA was substantially prolonged (Fig. 6B; see Table S7 in the supplemental material). Thus, although RNase E is necessary for rapid degradation of monophosphorylated *rpsT* P1 and *yfcZ* mRNA, either RNase E or RNase G is sufficient to de-

grade the monophosphorylated *ydfG* intermediate at an adequate rate. These results are consistent with the conclusion, based on PABLO analysis, that the decay rates of *rpsT* P1 and *yfcZ* mRNA are limited by degradative events subsequent to pyrophosphate removal (i.e., by RNase E cleavage), whereas the decay rate of *ydfG* mRNA is not. In the absence of RNase E, *E. coli* appears to contain sufficient RNase G to degrade the monophosphorylated *ydfG* intermediate without a significant change in the overall rate of *ydfG* decay, which is governed by the rate of formation of that intermediate and not by its rate of breakdown. In contrast, the decay rates of the *rpsT* P1 and *yfcZ* transcripts are governed by cleavage of the monophosphorylated intermediate and are therefore dependent on RNase E availability.

Conclusions. These findings show that the rate of RppH-dependent RNA degradation in *E. coli* can be limited either by pyrophosphate removal or by subsequent RNase E cleavage. When pyrophosphate removal is rate determining, RNase G can be an effective substitute for RNase E in subsequent cleavage events despite its low abundance, whereas RNase E is critical for rapid decay when those cleavage events are rate determining. Furthermore, the influence of RppH on the phosphorylation state of *E. coli* transcripts degraded by a 5'-end-dependent mechanism indicates that they can be categorized into at least two groups: those, like *rpsT* P1 and *yfcZ*, for which the concentration of RppH limits the rate of pyrophosphate removal and those, like *ydfG*, for which it does not. The difference between the two groups of mRNAs may be quantitative rather than qualitative; for example, an RppH-independent event might trigger pyrophosphate removal from all transcripts that are targeted by RppH but might be rate limiting only for the latter group. Among the former group of transcripts, a triggering event that is just a few times faster than subsequent RppH-catalyzed pyrophosphate removal could help to explain why an ~250-fold increase in the concentration of soluble RppH increases the ratio of monophosphorylated to triphosphorylated *rpsT* P1 and *yfcZ* mRNA by only a factor of 3 to 7.

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