NAR Breakthrough Article

Reversible acetylation on Lys501 regulates the activity of RNase II

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ABSTRACT

RNase II, a 3′ to 5′ processive exoribonuclease, is the major hydrolytic enzyme in *Escherichia coli* accounting for ~90% of the total activity. Despite its importance, little is actually known about regulation of this enzyme. We show here that one residue, Lys501, is acetylated in RNase II. This modification, reversibly controlled by the acetyltransferase Pka, and the deacetylase CobB, affects binding of the substrate and thus decreases the catalytic activity of RNase II. As a consequence, the steady-state level of target RNAs of RNase II may be altered in the cells. We also find that under conditions of slowed growth, the acetylation level of RNase II is elevated and the activity of RNase II decreases, emphasizing the importance of this regulatory process. These findings indicate that acetylation can regulate the activity of a bacterial ribonuclease.

INTRODUCTION

Ribonucleases (RNases) play important roles in essentially all aspects of RNA metabolism including maturation of RNA precursors, turnover of mRNA, quality control of stable RNAs and degradation of stable RNAs under stress conditions (1–5). As components that rapidly modulate the level of RNAs, the expression of RNases must be tightly controlled so that cells can quickly respond to environmental changes. However, despite its importance, relatively little is known about the mechanisms of this control or how such changes might be regulated. One RNase known to be regulated is *Escherichia coli* RNase II, whose levels decrease dramatically under a variety of stress conditions, such as slowed growth and nitrogen deprivation (6). RNase II is a ubiquitous, processive exoribonuclease that degrades RNA in the 3′ to 5′ direction releasing 5′-nucleotide monophosphates (7,8). This protein, encoded by *rnb* gene, is the major hydrolytic enzyme in *E. coli*, accounting for 90% of the exoribonucleolytic activity in cell extracts (9,10). Hence, its regulation may be important for a cell’s response to stress conditions. Based on these considerations, understanding the mechanisms by which RNase II is regulated is of considerable interest.

The expression of RNase II can be controlled at different levels. Earlier studies showed that RNase II is expressed from two different promoters, P1 and P2 (11), suggesting transcriptional regulation of this protein. Inactivation of some ribonucleases, including RNase E, RNase III and PNPase, leads to altered levels of RNase II (12). In fact, RNase II levels can vary over a 5-fold range depending on the amount of PNPase present in the cell. Cells overproducing PNPase decrease *rnb* mRNA and RNase II, whereas cells deficient in PNPase have an increased amount of RNase II (13). The stability of RNase II is also post-translationally regulated. Deletion of a gene, termed *gmr*, which lies downstream of *rnb*, dramatically increases the stability and amount of this protein (6). Moreover, RNase II was shown to be associated with the cytoplasmic membrane through its NH2-terminal amphipathic helix, and this interaction is critical for the viability of the cells in the absence of PNPase (14). Recently, several proteomics studies included RNase II among over 782 acetylated proteins in exponential phase *E. coli* cells grown in rich or minimal medium (15–17). However, to date, the mechanism and role of this post-translational modification remain elusive.

Here, we show that RNase II is acetylated on at least one residue, Lys501, which is close to the catalytic center bind-
ing the first few 3’ nucleotides of the substrate. Addition of an acetyl group to Lys501 leads to decreased substrate binding and catalytic activity of this enzyme. Post-translational modification of RNase II is reversibly controlled by the acetyltransferase and deacetylase pair, Pka and CobB. Importantly, we find that under slowed growth conditions, the acetylation level of RNase II is elevated and elimination of this modification alters the growth rate of the cells possibly by affecting the degradation of ribosomal RNAs, confirming its physiological relevance. These findings provide a clear example in which acetylation regulates the activity, but not the stability, of an RNase in bacteria.

MATERIALS AND METHODS

Materials

Anti-FLAG M2 mAb, Anti-FLAG M2 Affinity Gel, FLAG peptide, trichostatin A (TSA) and nicotinamide (NAM) were from Sigma. Acetylated-lysine mouse mAb was purchased from PTM Biolabs. [5,3H]-uridine was purchased from PerkinElmer Life Sciences. His-probe (H3) monoclonal antibody and anti-mouse IgG HRP conjugate were obtained from Santa Cruz Biotechnology. RNeasy mini kit was from Qiagen. M-MLV reverse transcriptase and Rnasin were from Promega. Protease inhibitor cocktail was purchased from Calbiochem.

Preparation of RNase II mutant strains

Escherichia coli K12 strain MG1655(Seq)rph+ and its derivatives lacking RNase II, Pka or CobB were obtained from Dr. Kenneth Rudd, University of Miami (18). DNA encoding the 2xFLAG sequence was fused to the N-terminus of chromosomal RNase II following a previously published recombineering protocol (19) using oligos R1 and R2 (Supplementary Table S1). Recombinants were selected on LB-kanamycin plates. The kanamycin resistance cassette was obtained from pTiC58. The 2xFLAG peptide, trichostatin A (TSA) and nicotinamide (NAM) were added to final concentrations of 0.5 mM and 200 μM, respectively. The cultures were grown at 37°C, with and without antibiotics. Antibiotics, when present, were at concentrations of kanamycin, 50 μg/ml; chloramphenicol, 100 μg/ml; ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml. Exponential phase cells were collected at an A560 of ~0.3. For deacetylase inhibitor treatment, 10 μM of overnight grown culture was inoculated into 10 ml of YT medium and TSA and NAM were added to final concentrations of 0.5 mM and 5 mM, respectively. The cultures were grown at 37°C to an A550 of ~0.3 and then harvested.

Pulldown of RNase II

Cells were disrupted in binding buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 0.5% NP-40, 1 mM PMSF) containing protease inhibitor cocktail as described (19). After centrifugation at 12 000 × g for 15 min, the supernatant fraction was collected and one mg of soluble protein was incubated with 50 μl of anti-FLAG M2-agarose suspension pre-activated according to manufacturer’s instructions at 4°C for 2 h. After centrifugation, the beads were washed five times with 500 μl of binding buffer, and RNase II was eluted with 50 μl of binding buffer containing 1 mg/ml FLAG peptide.

RNase II activity assay

The activity of RNase II was determined as described (22). Assays were carried out in 50 μl reaction mixtures containing 50 mM Tris–HCl (pH 8.0), 300 mM KCl, 0.25 mM MgCl2, 5 mM DTT and 30 μg [3H]polyadenylic acid substrate (100 cpm nmol-1). The concentration of purified RNase II or cell extracts were adjusted to ensure that less than 25% of the substrate was degraded. Reaction mixtures were incubated at 37°C for 15 min. The reaction was stopped by the addition of 150 μl of 0.5% (w/v) yeast RNA and 200 μl of 20% (w/v) trichloroacetic acid. The mixture was incubated on ice for 15 min and then centrifuged at 16 000 × g for 10 min at 4°C. The acid-soluble counts in 200 μl of the supernatant fraction were determined by liquid scintillation. Activities were normalized according to the moles or mg of protein in each sample.

Binding assay

The double-filter nucleic acid-binding assay was performed as described (22). Nitrocellulose and nylon membranes were washed and equilibrated in binding buffer (50 mM Tris–HCl, pH 8.0, 100 mM KCl, 5 mM DTT, 10 mM ethylenediaminetetraacetic acid (EDTA) and 10% glycerol) for at least 1 h prior to use. Twenty microliter reaction mixtures containing 50 mM Tris–HCl (pH 8.0), 100 mM KCl, 5 mM DTT, 10 mM EDTA, 10% glycerol, 200 pM 32P-labeled oligoribonucleotide substrate and varying amounts of purified RNase II enzyme were incubated on ice for 30 min. No degradation of the RNA occurs in the absence of Mg2+. A 96-well dot-blot apparatus (Bio-Rad) was used to apply the sample to a nitrocellulose membrane placed above a nylon membrane. Each well was washed with 100 μl of ice-cold binding buffer just prior to loading the sample and immediately following sample application. The apparatus was disassembled, the membranes were allowed to air dry and were visualized using a PhosphorImager. Quantification was carried out in ImageJ, and the Kd was determined using nonlinear regression analysis in GraFit 4 software (Eyrathicus Software).

Measurement of RNase II half-life

Cells were grown in YT medium to an A550 of ~0.3. A portion of the culture was collected for the zero time point and chloramphenicol was added to the remaining culture at 200 μg/ml. Cells were collected at the indicated times, lysed by sonication (23) and assayed by immunoblotting to determine the amount of RNase II remaining using anti FLAG antibody.
Purification of RNase II

pET-**rnb** was transformed into BL21 (DE3) cells lacking Pka or CobB for the expression of non-acetylated and acetylated RNase II proteins, respectively. His-RNase II was overexpressed and purified as previously described (22).

Pka-mediated *in vitro* acetylation

The *in vitro* reaction was performed as described (24) with 50 ng purified WT, K501R or K501Q RNase II proteins in the absence or presence of 50 ng purified Pka in a volume of 20 μl. Reaction mixtures were incubated at 37°C for 1 h and then analyzed by immunoblotting using acetylated-lysine antibody or anti FLAG antibody.

CobB-mediated *in vitro* deacetylation

The *in vitro* deacetylation reaction was carried out as described (24) with 50 ng of purified WT, K501R or K501Q RNase II proteins and 50 ng of purified His-CobB in a volume of 20 μl. Reaction mixtures were incubated at 37°C for different times, and analyzed by immunoblotting using acetylated-lysine antibody or anti FLAG antibody.

Western blot analysis

Proteins were resolved on an 8% gel and subjected to immunoblotting. FLAG-RNase II, acetylated RNase II, His-RNase II were detected by anti-FLAG M2 mAb (1:1000 dilution), acetylated-lysine mouse mAb (1:1000 dilution) and His-probe monoclonal antibody (1:1000 dilution), respectively. Underexposed films were used for quantification by Quantity One (Bio-Rad).

qRT-PCR analysis

RNA was extracted from cells with the RNeasy mini kit according to the manufacturer’s protocol. For qRT-PCR, 2 μg of total RNA was used for first-strand cDNA synthesis by M-MLV in the presence of primer Y1 (for *yfiA*), O1 (for *ompA*) or U1 (for *udp*) (Supplementary Table S1). qRT-PCR analysis was performed as described previously (25). Expression levels of these genes were normalized to those of the *E. coli* 16SrRNA gene. Primers Y1 and Y2, O1 and O2, and U1 and U2 were used to quantify gene expression levels of *yfiA*, *ompA* and *udp*, respectively (Supplementary Table S1).

Growth competition

Equal amounts of wild-type (WT) (Kan^R^) and K501R or K501Q mutant strain cells, based on absorbance, were mixed and diluted into M9/0.2% glucose medium. The culture was incubated at 37°C with constant shaking at 200 rpm. Growth was monitored by A_{550} measurements. After 6 h, the culture, still in exponential phase growth, was diluted 1:1000 into fresh M9/0.2% glucose phase medium. This represents one cycle (6 h) of exponential growth competition. Such cycles were repeated 3 times. Before each cycle, 100 μl of culture was taken, diluted and plated onto YT plates with or without kanamycin. The CFU for each strain was determined at the beginning and end of each cycle. Calculation of the strains’ doubling time was carried out as previously described (26).

*In vivo* assay for ribosome degradation

A single colony was inoculated into 2 ml of M9/0.2% glucose medium. After overnight growth, 100 μl was inoculated into 100 ml of M9/0.2% glucose supplemented with 1 mCi/ml of [3H]-uridine (GE) and 0.1 mM uridine. Cultures were grown to mid-exponential phase and collected by centrifugation for 10 min. The cell pellet was washed once in M9 salts and resuspended in 50 ml of M9 salts and 0.1 mM uridine. After 4 h incubation, 500 μl portions were removed from each culture and treated with 4 M formic acid (27). After 15 min on ice, samples were centrifuged at 13 000 rpm for 15 min in a Fisher bench top microcentrifuge at 4°C. Half of the supernatant fraction was removed and neutralized with 1 M Tris. Ten milliliters of scintillation fluid was added, and samples were counted in a scintillation counter to determine acid-soluble radioactivity.

RESULTS

RNase II is acetylated on lysine 501

Several acetylation proteomics studies identified RNase II as a putative substrate of acetylation (15–17). To confirm this observation, 2×FLAG was fused to the N terminus of RNase II in the chromosome and the fusion protein was pulled down with anti FLAG agarose beads. Using an antibody directed against N-acetyl-lysine, we examined the modification status of RNase II and found that this protein was indeed acetylated (Figure 1A). The cells also were treated with NAM, an inhibitor of the SIRT family deacetylase, and TSA, an inhibitor of class I and class II histone deacetylase, respectively. The results showed that the acetylation level of RNase II was enhanced ~3.0-fold after treatment with NAM (Figure 1A), whereas the addition of TSA had little effect on the modification of this enzyme, indicating that the acetylation of RNase II was regulated by a SIRT family deacetylase.

Lys501 in RNase II was found to be potentially acetylated in previous acetylome studies (15–17). To identify whether Lys501 is indeed acetylated, we mutated lysine 501 to arginine (R) or glutamine (Q) in the chromosome with 2×FLAG at its N-terminus, respectively, and examined their acetylation using the anti-acetyllysine antibody. Note that arginine and glutamine mimic non-acetylated and acetylated lysine, respectively, with respect to charge on the residues. Our results showed that mutation of Lys501 resulted in up to 80% reduction in acetylation of RNase II (Figure 1B) indicating first, that under these conditions, Lys501 is the major site of acetylation in this enzyme and second, that some other lysine residues in RNase II are also susceptible to acetylation. To further confirm the acetylation of RNase II Lys501, WT and mutant strains also were treated with NAM, and then the acetylation of RNase II in these cells was measured. As shown in Figure 1B, after NAM treatment, the acetylation level of RNase II increased 2–3-fold in both the WT and the mutant strains. Therefore,
processively on single-stranded RNA in the 3′-end of the acid-precipitable polymer chain is monitored. The activity on poly(A) was determined using an acid soluble AMP assay as described in ‘Materials and Methods’ section. In this assay the release of acid-soluble AMP molecules from the acetylated WT enzyme. By qRT-PCR, the amount of preexisting RNase II was determined the amount and half-life of the mutant RNase II and those presented above, we conclude that Lys501 acetylation decreases RNase II activity.

**Acetylation decreases RNase II activity**

Regulation of enzymes by acetylation of lysine occurs by various mechanisms (24,28). As Lys501 is close to the catalytic center of RNase II (22,29), acetylation on this residue might have an effect on its enzymatic activity. To test this hypothesis, the total activity of RNase II in the cell was measured using poly(A) as substrate (22). As RNase II acts processively on single-stranded RNA in the 3′ to 5′ direction releasing adenosine monophosphate (AMP) molecules, activity on poly(A) was determined using an acid soluble assay as described in ‘Materials and Methods’ section. In this assay the release of acid-soluble AMP molecules from the acid-precipitable polymer chain is monitored. The activity of WT RNase II decreased ∼50% after NAM treatment (Figure 2A) in concert with the elevated acetylation level of this enzyme (Figure 1B). To determine the specific effect of Lys501 acetylation, we measured the activity of WT and mutant RNase II proteins in the presence or absence of NAM. As shown in Figure 2A, the K501R mutant displayed activity ∼2-fold higher than the WT RNase II, whereas the K501Q mutant behaved very similarly to the acetylated WT enzyme. In contrast to the WT RNase II that was partly inactivated by NAM, a similar treatment by this deacetylase inhibitor had little effect on the activity of either K501R or K501Q mutant proteins (Figure 2A), indicating that acetylation of Lys501 is responsible for the reduced activity of RNase II.

To further investigate the effect of lysine acetylation on RNase II activity in vivo, we determined the level of three mRNAs, *yfiA*, *ompA* and *udp*, the known targets of this enzyme, by qRT-PCR. *yfiA* is a substrate of RNase II, and inactivation of this enzyme increases the steady-state level of *yfiA* ∼3-fold (30; Figure 2B). Consistent with the *in vitro* results, the level of *yfiA* was reduced by 40% in the K501R mutant, whereas the amount of this message increased ∼2-fold in K501Q cells (Figure 2B). Although *yfiA* was elevated ∼2-fold in WT cells after NAM treatment (Figure 2B) due to its increased acetylation level (Figure 1B), this was not observed in the K501R or K501Q mutant strains in which the acetylation does not occur (Figure 2B). Besides its role in RNA degradation, RNase II also stabilizes some mRNAs, including *ompA* and *udp*, through the removal of their poly(A) tails that can be used as binding sites by other RNases (30,31), such as RNase R and PNPase (31). In contrast to *yfiA*, substitution of Lys501 with arginine increased the amount of *ompA* and *udp* by ∼50%, while mutation of this residue to glutamine reduced the level of these two messages (Figure 2C and D). Again, the presence of NAM had little effect on the expression level of *ompA* and *udp* in the mutant strains (Figure 2C and D). Based on these results and those presented above, we conclude that Lys501 acetylation decreases RNase II activity.

**Acetylation affects the binding activity of RNase II**

To assess the contribution of RNase II acetylation to substrate binding, a double-filter binding assay was used to determine the *Kd* for binding of single-stranded A17 to different forms of RNase II. Binding assays were carried out in the absence of Mg2+ and in the presence of EDTA to prevent degradation of the RNA upon binding to RNase II. As shown in Table 1, A17 bound tightly to WT (not acetylated, Figure 3A) RNase II with a *Kd* of 8.9 nM. This is consistent with previous data (22,32). A17 also bound tightly to the K501R mutant with a *Kd* similar to that of the WT form. In contrast, the acetylated protein and the K501Q mutant bound A17 with *Kd* values ∼4-fold higher than the WT RNase II (Table 1), indicating that acetylation of Lys501 exerts a negative effect on substrate binding.

**Effect of acetylation on RNase II stability**

Recent studies revealed that RNase R is an extremely unstable protein in exponential phase cells due to acetylation on a single amino acid residue, Lys544 (21,33). To examine whether acetylation might also affect RNase II levels, we determined the amount and half-life of the mutant RNase II proteins, K501R and K501Q. WT and K501R and K501Q mutant strain cells growing in the exponential phase were treated with chloramphenicol to inhibit new protein synthesis, and the amount of preexisting RNase II was deter-
Figure 2. Activity of RNase II in WT and mutant strains. (A) RNase II activity in WT, K501R and K501Q mutant strains with or without NAM treatment. The enzyme activity against poly(A) was determined with 1 µg of soluble proteins as described in ‘Materials and Methods’ section for 30 min at 37°C. The data presented are the average of three independent experiments in which the activity of RNase II in the WT cells without NAM treatment was set at 1. (B) Relative yfiA level in WT, K501R, K501Q and rnb mutant strains with or without NAM treatment. (C) Relative ompA level in WT, K501R, K501Q and rnb mutant strains with or without NAM treatment. (D) Relative udp level in WT, K501R, K501Q and rnb mutant strains with or without NAM treatment. For (B and D), the average of three independent experiments is shown in which the level of yfiA, ompA and udp in the WT cells without NAM treatment was each set at 1.

Table 1. RNA binding to RNase II proteins

<table>
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<tr>
<th>Protein</th>
<th>WT</th>
<th>Ac-K</th>
<th>K501R</th>
<th>K501Q</th>
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<tr>
<td>KD (nM)</td>
<td>8.9 ± 1.1</td>
<td>35.1 ± 2.3</td>
<td>8.8 ± 1.0</td>
<td>34.6 ± 2.2</td>
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The KD values were determined by a filter binding assay as described in ‘Materials and Methods’ section. Each value represents the mean of three experiments. Ac-K indicates the acetylated form of RNase II. WT (not acetylated) and acetylated RNase II proteins were purified from BL21 cells lacking Pka or CobB, respectively.

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Acetylation of RNase II by Pka

To examine the relation between acetylation of Lys501 and RNase II activity in more detail, it was of interest to identify and eliminate the enzyme responsible for acetylation of the RNase. Although E. coli contains multiple genes that may encode the putative acetyltransferase (34), we initially focused on Pka, the product of the pka gene. Pka is the only acetyltransferase identified so far in E. coli, and is responsible for acetylation of RNase R (21), an ortholog of RNase II. In addition, Pka is highly similar to the Pat protein of Salmonella which was shown to participate in acetylation of multiple proteins in that organism (24). Accordingly, we determined the acetylation level of RNase II in a strain in which the pka gene is interrupted with a kanamycin cassette. Removal of Pka completely eliminates acetylation of RNase II in both the WT and the K501R and K501Q mutant strains (Figure 3A, top panel), indicating that Pka is the enzyme which acetylates Lys501 and other lysine residues. Concomitantly, the amount of yfiA is reduced by 50% in WT cells, but is not changed in K501R and K501Q mutant strains (Figure 3B). To determine whether Pka can directly modify RNase II, WT and mutant RNase II proteins purified from BL21 cells lacking Pka, which are not acetylated (Figure 3C), were incubated with purified Pka in the presence of acetyl-CoA, the acetyl group donor. As shown in Figure 3C, purified Pka acetylates WT RNase II effectively in vitro, whereas it displays low activity against either the K501R or the K501Q mutant RNase II protein (Figure 3C). In agreement with the in vivo results, Pka treatment dramatically decreased the activity against poly(A) of the WT protein, but had no obvious effect on the K501R and K501Q
Figure 3. Acetylation of RNase II by Pka. (A) Acetylation (Ac-K, top panel) and amount (bottom panel) of RNase II in WT, K501R and K501Q mutant strains in the absence or the presence of Pka. Cells were lysed and subjected to immunoprecipitation with anti FLAG antibody agarose beads. Precipitates were then analyzed by immunoblotting using anti acetylated-lysine monoclonal antibody or anti-FLAG monoclonal antibody. Shown are representative gels from experiments carried out twice. The amount of acetylated and total RNase II in WT cells with Pka was each set at 1. (B) Relative yfiA level in WT, K501R and K501Q mutant strains in the absence or the presence of Pka. The data presented are the average of three independent experiments in which the level of yfiA in the WT cells in the presence of Pka was set at 1. (C) Pka directly acetylates RNase II in vitro. Purified WT, K501R or K501Q mutant RNase II proteins (50 ng) were incubated with or without 50 ng of purified Pka. Products were then analyzed by immunoblotting using anti acetylated-lysine monoclonal antibody or His-probe monoclonal antibody. Each gel shown is a representative experiment carried out twice. The amount of acetylated and total RNase II in the reaction of WT protein with Pka treatment was each set at 1. (D) RNase II activity of WT, K501R and K501Q mutant RNase II with or without Pka treatment. The enzyme activity was determined as described in Figure 2. The data presented are the average of three independent experiments in which the activity of WT RNase II without Pka treatment was set at 1.

Deacetylation of RNase II by CobB

Lysine acetylation is a reversible post-translational modification that is regulated by acetyltransferases and deacetylases. Because NAM, but not TSA, significantly increased RNase II acetylation, we speculated that a SIRT family deacetylase is involved in deacetylation of this enzyme. CobB plays a major role in deacetylation in bothSalmonellaandE. coli(17,24), and is the only known protein deacetylase in the latter organism (35). Thus, we examined the effect of removing the product of thecobBgene. Not unexpectedly, inactivation of CobB increased the acetylation of WT RNase II (Figure 4A) leading to decreased activity againstyfiAin vivo(Figure 4B). Moreover, using RNase II purified fromcobBmutant BL21 cells, we found that purified CobB can effectively remove acetyl groups from RNase II in vitro(Figure 4C). Given the fact that the same amounts of RNase II and CobB were used in this assay, we conclude that CobB can act on all acetylated lysine residues including Lys501. Likewise, this treatment increased the activity of RNase II ∼3.5-fold(Figure 4D). Although CobB is also able to act on other acetylated lysine residues besides Lys501, these changes of acetylation levels have no effect on its activity(Figure 4B and D). Collectively, these data establish a specific and prominent role of CobB in RNase II deacetylation and enzymatic activation.

Elevation of RNase II acetylation during slowed growth

Increasing evidence suggests that metabolic pathways are coordinated with reversible acetylation of metabolic enzymes in response to the level of nutrients(24,36). The level of RNase II is known to change in response to growth conditions such as slowed growth in which the amount of this enzyme decreases dramatically (6). To determine whether acetylation of RNase II is dynamically regulated in vivo, we investigated the effect of slowed growth on RNase II modification. WT, K501R and K501Q mutant strains were cultured in YT and M9/glucose media, and the level of RNase II acetylation and RNase II activity were measured. Under these conditions, WT cells grow with doubling times of 30 and 75 min, respectively. As shown in Figure 5A, slowed growth increased the acetylation level of WT RNase II by 3.1-fold, and as a result, its activity was reduced about 50% (Figure 5B). Notably, slowed growth had little effect on the activity of the K501R and K501Q mutants (Figure 5B), although it increased their acetylation level slightly (Figure...
Deacetylation of RNase II by CobB. (A) Acetylation (Ac-K, top panel) and amount (bottom panel) of RNase II in WT, K501R and K501Q mutant strains in the absence or the presence of CobB. The experiments were carried out as in Figure 3. Shown are representative gels from experiments carried out at least twice. The amount of acetylated and total RNase II in wild type cells with CobB was each set at 1. (B) Relative yfiA level in WT, K501R or K501Q mutant RNase II proteins (50 ng) were incubated with or without 50 ng of purified CobB and then analyzed by immunoblotting using anti acetylated-lysine monoclonal antibody or His-probe monoclonal antibody. Each gel shown is a representative experiment carried out twice. The amount of acetylated and total RNase II in the reaction of WT protein without CobB treatment was each set at 1. (C) CobB directly deacetylates RNase II in vitro. Purified WT, K501R or K501Q mutant RNase II proteins (50 ng) were incubated with or without 50 ng of purified CobB and then analyzed by immunoblotting using anti acetylated-lysine monoclonal antibody or His-probe monoclonal antibody. Each gel shown is a representative experiment carried out twice. The amount of acetylated and total RNase II in the reaction of WT protein without CobB treatment was each set at 1. (D) RNase II activity of WT, K501R and K501Q mutant RNase II with or without CobB treatment. The enzyme activity was determined as described in Figure 2. The data presented are the average of three independent experiments in which the activity of WT RNase II without CobB treatment was set at 1.

Since *E. coli* has evolved such an extensive regulatory process to modulate the activity of RNase II, it is likely that elevated acetylation of this enzyme is physiologically important for cells growing under low nutrient conditions. To examine this point in more detail, we determined the doubling time of WT cells and the K501R and K501Q mutant strains. Based on competition experiments, we found that while the K501Q mutant grew very similarly to WT cells, which are now largely acetylated (Figure 5A), the K501R mutant grew somewhat more slowly (Figure 5C).

One possibility to explain this slower growth is that the degradation of important RNA molecules such as ribosomal RNAs (rRNAs) may have changed. rRNAs, which account for as much as 90% of the total RNA in *E. coli*, generally are stable in growing cells, but their degradation increases under conditions such as starvation (37). Therefore, we determined the degradation of rRNAs in WT and K501R and K501Q mutant cells during starvation using a previously described *in vivo* assay (37). To do so, cells were grown in M9/glucose medium supplemented with [3H]-uridine to label ribosomes. The labeled cells were then resuspended in M9 salts without glucose, and after 4 h incubation, the degradation of pre-labeled ribosomes during this starvation period was measured. As shown in Figure 5D, substitution of arginine for lysine reduced the amount of acid-soluble material produced ∼50%, confirming that acetylation on Lys501 plays an important role in the degradation of rRNAs during starvation. Taken together, all of these data strongly suggest a role for RNase II Lys501 acetylation in modulating cell growth during stress conditions.

**DISCUSSION**

RNases are major participants in essentially all aspects of RNA metabolism. Yet, they are also destructive enzymes that potentially could cause serious problems with a cell’s complement of RNA (38). Therefore, cells must elaborately control RNase activities to ensure that elimination of functionally important RNA molecules is avoided. Although known for many years, the study of RNase regulation has until recently received relatively little attention (39). The information presented here indicates that one important exoribonuclease, RNase II, can be regulated by post-translational modification. Moreover, the modification does not affect the enzyme’s stability, as is the case for RNase R, but, rather, the protein’s catalytic activity. As such, these findings greatly expand our understanding of RNase regulation and open up new possibilities for further investigations in the RNase field.
The studies described here provide evidence that lysine acetylation can regulate the activity of an important enzyme and thus the cell’s ability to adapt to stress conditions. Specifically, we found that (i) RNase II, the predominant activity against poly(A), is acetylated on at least one residue, Lys501; (ii) addition of an acetyl group on Lys501 affects substrate binding of RNase II leading to decreased activity of this enzyme; (iii) acetylation of RNase II is reversibly controlled by the acetyltransferase, Pka, and the deacetylase, CobB; (iv) slowed growth increases the acetylation level of RNase II; and (v) inhibition of Lys501 acetylation in RNase II lead to a slower growth rate likely due to altered degradation of RNAs. These findings, and others presented earlier (6,11–14), indicate that bacterial cells employ a variety of mechanisms to carefully control the amount, activity, specificity and cellular localization of RNase II. This complex regulatory system serves as a detailed example of how cells deal with stress conditions through regulation of an RNase.

RNase II action is highly processive on single-stranded RNA (ssRNA) which binds the substrate through two binding sites: a catalytic site associated with the first few 3′ nucleotides and an anchoring site ∼15–25 nt upstream of the 3′ end (22,40). Lys501 is within the catalytic center, and thus, modification of this residue might be expected to have an effect on substrate binding. Looking at the Lys501 position, acetylation will only partially block the RNA channel. The closest distance from the tip of Lys501 to the A12 phosphate backbone is around 5 Å (Supplementary Figure S2), so there is enough space to accommodate acetylation. In fact, the main effect is likely due to the change of charge. Addition of an acetyl group will neutralize the positive charge on Lys501 and thus reduce binding at the −1 position (one residue 5′ of the cleavage site, which is A13 in the structure) (Supplementary Figure S2). This hypothesis is supported by the observations that the K501R and K501Q mutant proteins behave similarly to the unmodified and acetylated RNase II proteins (Figure 2 and Table 1), respectively.

Protein acetylation is a reversible process via the control of acetyltransferase and deacetylase (24). We show here that acetylation of RNase II is reversibly controlled by Pka and CobB, the only acetyltransferase and deacetylase identified so far in E. coli, respectively (21,35). Treatment with NAM, an inhibitor of the SIRT family deacetylase, elevated RNase II acetylation ∼3-fold (Figure 1), suggesting that only one third of this enzyme is modified in growing cells. Mutation of Lys501 decreased the acetylation of RNase II ∼80%, indicating the presence of other modification sites in this protein. In agreement with these observations, recent proteomics analyses revealed that, besides Lys501, RNase II was acetylated on several other residues including Lys31, 68 and 107 (15–17), all of which are located in the N-terminal cold shock domain of this enzyme (22). This domain and the C-terminal S1 domain come together in a clamp-like ar-
rangement, which can only accommodate ssRNA (22). Although the role of acetylation on these three lysine residues are unclear, these modifications are likely to affect RNA binding based on their locations. The importance of these modifications needs to be further explored in future studies.

Besides enzyme-dependent acetylation, proteins can also undergo non-enzymatic modification by acetyl groups, which are quantitatively more numerous under appropriate conditions such as in glucose overloaded medium. The observations of Lys501 acetylation in this study but not in the experiments carried out by Schilling et al. (41) indicate that modification on this residue is enzyme-dependent and is not susceptible to non-enzymatic acetylation by acetyl-phosphate. Interestingly, acetylation of other RNases and helicases, including three components of the RNA degradosome, was detected in the latter research, suggesting that these modifications are largely dependent on acetyl-phosphate. Therefore, protein acetylation will change under different growth conditions with altered mechanisms.

The acetylation of RNase II is elevated under slowed growth conditions. In addition, inhibition of this modification by substitution of Lys501 with arginine leads to a slower growth rate of the cells. It has been suggested that the absence of RNase II results in increased degradation of rRNAs because the single-stranded 3' ends are stabilized allowing other RNases to bind more efficiently (37). These findings and our data suggest a model in which the slower growth of the K501R mutant cells can be attributed, at least partially, to an altered degradation rate for rRNAs. Inasmuch as RNase II is largely acetylated during slowed growth (Figure 5A), it would bind more weakly (Table 1). This decreased activity after acetylation, together with the reduced level of RNase II (6), would help to maintain the single-stranded 3' ends of rRNAs. As such, RNase R and PNPase would be able to bind more effectively and act on these RNA molecules during slowed growth. In the K501R mutant cells, RNase II binds more tightly to the 3' ends of rRNAs (Table 1), leading to decreased degradation by RNase R and PNPase. Although consistent with the data, additional experiments will be needed to conclusively prove this proposed model.

These studies also provide a clear example in which acetylation affects the activity of an RNase in bacteria. Acetylation has been shown to affect many proteins and processes in both prokaryotic and eukaryotic systems (36,42–44), but to date, only a few instances of a specific effect on a ribonuclease have been uncovered (21,33). The discovery here that acetylation of one specific lysine residue in RNase II decreases its catalytic activity, resulting in an altered growth rate, provides a clear example of a direct effect of acetylation on a regulatory process in bacteria. With the recent discovery that a large number of E. coli RNases are potentially acetylated in vivo (15–17,41), it is likely that the findings reported here are only the beginning of what will be a widespread phenomenon in bacteria.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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