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RNase III-Independent Autogenous Regulation of *Escherichia coli* Polynucleotide Phosphorylase via Translational Repression

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ABSTRACT

The complex posttranscriptional regulation mechanism of the *Escherichia coli pnp* gene, which encodes the phosphorolytic exoribonuclease polynucleotide phosphorylase (PNPase), involves two endoribonucleases, namely, RNase III and RNase E, and PNPase itself, which thus autoregulates its own expression. The models proposed for *pnp* autoregulation posit that the target of PNPase is a mature *pnp* mRNA previously processed at its 5′ end by RNase III, rather than the primary *pnp* transcript (RNase III-dependent models), and that PNPase activity eventually leads to *pnp* mRNA degradation by RNase E. However, some published data suggest that *pnp* expression may also be regulated through a PNPase-dependent, RNase III-independent mechanism. To address this issue, we constructed isogenic Δ*pnp rnc*$^+$ and Δ*pnp* Δ*rnc* strains with a chromosomal *pnp-lacZ* translational fusion and measured β-galactosidase activity in the absence and presence of PNPase expressed by a plasmid. Our results show that PNPase also regulates its own expression via a reversible RNase III-independent pathway acting upstream from the RNase III-dependent branch. This pathway requires the PNPase RNA binding domains KH and S1 but not its phosphorolytic activity. We suggest that the RNase III-independent autoregulation of PNPase occurs at the level of translational repression, possibly by competition for *pnp* primary transcript between PNPase and the ribosomal protein S1.

IMPORTANCE

In *Escherichia coli*, polynucleotide phosphorylase (PNPase, encoded by *pnp*) posttranscriptionally regulates its own expression. The two models proposed so far posit a two-step mechanism in which RNase III, by cutting the leader region of the *pnp* primary transcript, creates the substrate for PNPase regulatory activity, eventually leading to *pnp* mRNA degradation by RNase E. In this work, we provide evidence supporting an additional pathway for PNPase autogenous regulation in which PNPase acts as a translational repressor independently of RNase III cleavage. Our data make a new contribution to the understanding of the regulatory mechanism of *pnp* mRNA, a process long since considered a paradigmatic example of posttranscriptional regulation at the level of mRNA stability.

A wealth of mechanisms that control gene expression and an intricate network of regulatory interactions subtly and promptly adapt the presence and concentration of gene products to a variety of environmental and developmental conditions. Autogenous regulation of the *pnp* gene in *Escherichia coli* has long since been considered an example of regulation at the level of mRNA stability. This gene codes for polynucleotide phosphorylase (PNPase), a phosphorolytic 3′-to-5′ exoribonuclease and a template-independent, nucleoside diphosphate-dependent RNA polymerase that is conserved in bacteria and eukaryotic organelles (1, 2). *E. coli* PNPase plays a major role in RNA turnover and metabolism (3) and has been implicated in several processes, such as adaptation and growth in the cold, biofilm formation, and responses to oxidative stress and DNA damage (4–8).

Early studies showed that *pnp* belongs to two overlapped operons transcribed from the P1 (upstream from *rpsO*) and P2 (upstream from *pnp*) promoters (9–12). Both of the *pnp*-encoding mRNAs generated from P1 and P2 are efficiently processed by RNase III at a hairpin in the *pnp* untranslated leader region (UTR) between P2 and the *pnp* UUG start codon (Fig. 1). In the absence of RNase III, the primary transcripts are stable and efficiently translated, whereas upon RNase III processing, *pnp* mRNA is rapidly degraded and PNPase production ceases (13, 14). However, in the absence of PNPase, both RNase III-processed and unprocessed *pnp* mRNAs are stable (15). These observations led to the conclusion that PNPase regulates its own expression in an RNase III-dependent (RTD) manner.

Two basic alternative models have been proposed by Portier and coworkers to explain how PNPase regulates its own expression upon RNase III cleavage. A former model (15) essentially postulated that PNPase could act as a translational repressor by binding determinants (translational operators) in the 5′-UTR of the RNase III-processed *pnp* mRNA, thus promoting degradation of the untranslated mRNA by RNases other than PNPase. In contrast, in the primary transcript, the translational operator could not be available to PNPase; as a consequence, the *pnp* mRNA could be translated and thus stabilized. This model was supported by the observation that the mRNA stability of a *pnp-lacZ* transla-

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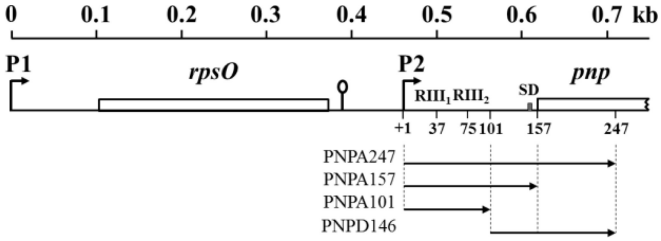

FIG 1 Genetic organization of the *E. coli pnp* regulatory region. The scale at the top corresponds to the MG1655 reverse genomic sequence, positions 3309850 to 3309100 (EMBL accession number AE000397). The bottom scale refers to the transcript from the P2 promoter. P1 and P2, promoters; lollipop, t1 transcription terminator; $RIII_1$ and $RIII_2$, RNase III cut sites; SD, Shine-Dalgarno region. The arrows represent the RNA probes used in this study.

tional fusion correlated inversely with ectopically expressed PNPase abundance, whereas it decreased in the presence of mutations affecting its translation efficiency (15).

A later model (16) was based on the observation that an RNase III double-strand cut generates a processed mRNA with a double-stranded stem in which the 5′-monophosphate recessed end is protected by the dangling 3′ end of a short RNA. It was then proposed that the processed mRNA with a 5′-end duplex would maintain the stability and translational properties of the primary transcript; on the other hand, PNPase could bind such a structure and degrade the short upstream strand of the duplex, thus releasing a stemless processed *pnp* mRNA. The *pnp* mRNA devoid of its 5′-end hairpin would become unstable and poorly, if at all, translatable, regardless of the intracellular PNPase concentration, as shown *in vivo* by these authors. In both models, autogenous regulation is exerted downstream from the RNase III cleavage step and leads, eventually, to *pnp* mRNA instability.

Within this framework, we previously showed that the RNase III-processed *pnp* mRNA that is devoid of the RNase III hairpin at its 5′ end is not translatable and is degraded by RNase E in a PNPase-independent manner (17). It thus appears that, upon RNase III cleavage, PNPase simply degrades the short 5′ complementary strand and is not further implicated in PNPase mRNA instability or translational repression. However, some previously published data may lend some support to a PNPase-dependent, RNase III-independent (RTI) regulatory mechanism, as well as translational repression by PNPase (see Discussion) (14, 15).

In this work, we provide evidence that PNPase also regulates its own expression via an RTI pathway. This pathway requires the PNPase RNA binding domains KH and S1 but not its phosphorolytic activity and operates upstream from the RTD pathway. We suggest that the RTI autoregulation of PNPase occurs at the level of translational repression, possibly by competition between PNPase and the ribosomal protein S1 for the *pnp* mRNA.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacteria, plasmids, and phages are described in Table 1, with a brief outline of their construction by standard techniques. Unless otherwise stated, bacterial cultures were grown at 37°C in LD medium (24). Where indicated, 2 g/liter arabinose, 2 g/liter glucose, 50 μg/ml ampicillin, and 30 μg/ml chloramphenicol were added.

Enzymes and reagents. Wild-type PNPase purification and anti-PNPase polyclonal antibodies have been previously described (25, 26). Purified ribosomal protein S1 and anti-S1 antibodies were a generous gift of Udo Bläsi.

PNPase autoregulation and RNA binding assays. Bacterial strains harboring λGF2 prophage (*pnp-lacZ* translational fusion) and pBAD24

TABLE 1 Bacteria, plasmids, and phages used in the study

Strain, plasmid, or phage	Relevant description	Reference and/or source
Strains		
C-1a	*E. coli* strain C, prototrophic	18
C-5684	Δ*rnc-38*::*kan*	17
C-5691	Δ*pnp-751*	19
C-5691(λGF2)	Δ*pnp-751* (λ *pnp-lacZ*)	Derived from C-5691 by lysogenization with λGF2 at 37°C
C-5979	Δ*pnp-751* Δ*rnc-38*::*kan*	Derived from C-5691 by transduction with P1 HFT grown on C-5684
C-5979(λGF2)	Δ*pnp-751* Δ*rnc-38*::*kan* (λ *pnp-lacZ*)	Obtained by lysogenization with λGF2 at 37°C
DH10B	Recipient strain for transformation by electroporation with new plasmid constructs	20
GF5322	*recA*::Tn*10 pnp*::Tn*5* (λ *pnp-lacZ*)	14
Plasmids		
pAZ101	pGZ119HE-*pnp*$^+$; Cam^r	21
pAZ1112	pAZ101-*pnp*-S438A; Cam^r	25
pAZ133	pAZ101-*pnp*-ΔKHS1 (*pnp-833*); Cam^r	34
pBAD24	*araC araBp* ColE1; Amp^r	22
pBAD-pnp	pBAD24 *pnp*-His; Amp^r	4
pBAD-Pnp$^+$	pBAD24 *pnp*$^+$; Amp^r	BsiWI-HindIII fragment of pAZ101 cloned in pBAD-pnp
pBAD-pnpS438A	pBAD24 *pnp*-S438A; Amp^r	BsiWI-HindIII fragment of pAZ1112 cloned in pBAD-pnp
pBAD-pnpΔKHS1	pBAD24 *pnp*-ΔKHS1; Amp^r	BsiWI-HindIII fragment of pAZ133 cloned in pBAD-pnp
Phages		
P1 HFT	High frequency of transduction	Provided by R. Calendar; described in reference 23
λGF2	λ *pnp-lacZ* translational fusion from GF5322	14

TABLE 2 Oligonucleotides used in the study[a]

Primer	Sequence (5′→3′)[b]	RNA probe(s)[c]	Coordinates[d]
FG0676	**CTAATACGACTCACTATAGGG**ATGAATGATCTTCCGTTGC	PNPA247, PNPA157, PNPA101	3311326–3311308
FG0678	CAGCGGCAGTAGCCTGACGAGC	PNPA247, PNPD146	3311078–3311099
FG1387	AATGTAATATCCTTTCTCTTTCTTAG	PNPA157	3311167–3311192
FG1625	**CTAATACGACTCACTATAGGG**GGGTATTAACACCAGTGCCG	PNPD146	3311223–3311204
FG1710	GATCTTCTGCGCATCCTCGC	PNPA101	3311224–3311243

[a] Used as PCR primers with pAZ101 DNA as a template.
[b] Boldface letters correspond to the T7 promoter sequence.
[c] The PCR products were used as the template for T7 RNA polymerase transcription to obtain the RNA probes indicated.
[d] Coordinates are with reference to the sequence deposited in GenBank with accession number U00096.2.

derivatives expressing the different *pnp* alleles under the *araBp* promoter were grown overnight at 37°C in 5 ml LD glucose (*pnp* repressed), and 0.5 ml of the culture was centrifuged for 30 s at room temperature, resuspended in an equal volume of LD, and diluted 200-fold in 40 ml LD broth with glucose (*pnp* repressed) or arabinose (*pnp* expressed) at 37°C. The cultures were further incubated with shaking at 37°C to an optical density at 600 nm (OD_{600}) of 0.8; 10-ml samples were collected by centrifugation, resuspended in 0.5 ml TEDP (0.1 M Tris-HCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], and 1 mM phenylmethylsulfonyl fluoride [PMSF] protease inhibitor), and disrupted by sonication (two 30-s pulses at 40% amplitude). The samples were centrifuged for 15 min at 12,000 rpm at 4°C to remove cell debris. The protein concentration in the crude extracts was determined by using the Coomassie (Bradford) protein assay kit (Thermo Scientific). The β-galactosidase activity of the extracts was measured as described previously (27). Specific activity was expressed as nmol of *ortho*-nitrophenyl-β-D-galactopyranoside (ONPG) converted to *ortho*-nitrophenol min^{-1} mg $protein^{-1}$. The PNPase content of the samples was evaluated by Western blot analysis of 400-ng samples of total protein using anti-PNPase antibodies (28) and densitometric analysis of the film using ImageQuant software (Molecular Dynamics). Electrophoretic mobility shift assays (EMSAs) were performed as described previously (25). PNPase-RNA cross-linking assays were performed by incubating 100,000 cpm of the uniformly [α-^{32}P]CTP-labeled RNA probes (Fig. 1) for 20 min at 21°C in binding buffer (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.5 mM DTT, 0.025% NP-40 [Fluka], and 10% glycerol) with either 400 ng of crude extract or purified proteins in a final volume of 10 μl. The samples were UV irradiated (254-nm wavelength at 2.8 J/cm^2) and treated with RNase A, and the cross-linked proteins were fractionated by 10% SDS-PAGE and analyzed by phosphorimaging (28). RNA probes were obtained by T7 RNA polymerase transcription of DNA templates produced by PCR with the primers listed in Table 2 and plasmid pAZ101 as a template.

S1-PNPase binding assay. (i) Crude extract preparation. Amounts of 50 ml of exponential cultures (OD_{600} of 0.8) of strains C-1a/pAZ101and C-1a/pAZ133 were collected by centrifugation, resuspended in 0.35 ml buffer A (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$), and lysed by freeze-thawing with 0.4 mg/ml lysozyme. Then, 0.25 ml buffer A supplemented with 0.05% Tween and 0.1 U/μl DNase I (Promega) was added. After 20 min on ice, the extracts were centrifuged at 13,200 rpm for 10 min at 4°C. The absorbance at 260 nm was measured to get a rough estimate of crude extract concentration (29, 30). Where indicated below, the extracts were incubated for 20 min at 37°C with 250 ng/μl RNase A.

(ii) S1 coating of the beads and analysis of S1-PNPase binding. His-tagged S1 was purified with Ni-nitrilotriacetic acid (NTA)–agarose (Qiagen), following the manufacturer's protocol, from an exponential culture of strain C-1a/pREP4/pQE31-S1 (28) induced with 1 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Amounts of 25 μl of Ni-NTA–agarose beads (Qiagen) were washed with buffer A and incubated for 1 h at 4°C in a rotatory device with 150 pmol His-tagged S1 in 400 μl of 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 15 mM imidazole, 0.005% Tween. S1-coated beads were incubated for 1 h at 4°C in a rotatory device with 2 to 4 OD_{260} of crude extracts diluted in buffer B (10 mM Tris-HCl, pH 7.5, 280 mM NaCl, 20 mM imidazole; final volume, 500 μl). After incubation, the beads were washed twice with 500 μl buffer B. S1 was eluted by incubating the beads in 40 μl buffer C (10 mM Tris-HCl, pH 7.5, 30 mM NaCl, 250 mM imidazole). Proteins were run on 10% SDS-PAGE gels, and the gels were either silver stained with the SilverQuest silver staining kit (Invitrogen) or blotted onto a nitrocellulose (Hybond C) sheet and incubated with polyclonal anti-PNPase antibodies (26).

RESULTS

RNase III-independent autogenous regulation of PNPase. To monitor *pnp* operon regulation by PNPase, we used a previously described reporter system consisting of a translational fusion between the 5′ region of the *rpsO-pnp* operon, including the first 61 codons of *pnp* (Fig. 1), and the reporter gene *lacZ* carried by the transducing λGF2 phage (14, 31, 32). Single Δ*pnp* and double Δ*pnp* Δ*rnc-38* mutants were lysogenized with λGF2 and transformed by pBAD24 plasmid vector derivatives harboring *pnp* (or *pnp* mutants as described below) under the control of the arabinose-inducible promoter *araBp*. PNPase was expressed in the presence of arabinose (which induces transcription from *araBp*), whereas in the presence of glucose (*araBp* repression), as well as in the strains harboring the empty vector under either condition, no PNPase could be detected by Western blotting (data not shown).

Repression exerted by PNPase on the expression of the reporter *lacZ* was expressed as the ratio of β-galactosidase specific activity in the presence of glucose to that in the presence of arabinose. As shown by the results in Fig. 2, the induction of wild-type PNPase exerted, as expected, approximately 6-fold repression of β-galactosidase in the *rnc*$^+$ strain. In the Δ*rnc-38* mutant, however, repression was reduced to about 3- to 4-fold but not abrogated as would be predicted by the current autoregulation model. This result is consistent with data obtained by Portier and collaborators (14) in a different *E. coli* strain and with a similar system. It thus appears that PNPase participates in an RNase III-independent (RTI) mechanism of regulation of *pnp* operon expression.

RNase III-independent autogenous regulation requires RNA binding but not phosphorolytic activity. To test whether this residual RNase III-independent autogenous regulation required phosphorolytic, RNA binding, or both PNPase activities, we measured posttranscriptional repression levels by PNPase mutants affected in either activity, namely, Pnp-ΔKHS1, which is missing the two RNA binding domains (31, 33), and Pnp-S438A (with a mutation of S to A at position 438), which is devoid of phosphorolytic activity (25). To test whether the mutant retained RNA binding activity, we performed RNA-PNPase cross-linking experiments, as previously described (28). As shown by the results in Fig. 3A, the ratio of PNPase-bound RNA to PNPase, normalized to the wild-type PNPase signals, was not affected by the S438A mutation,

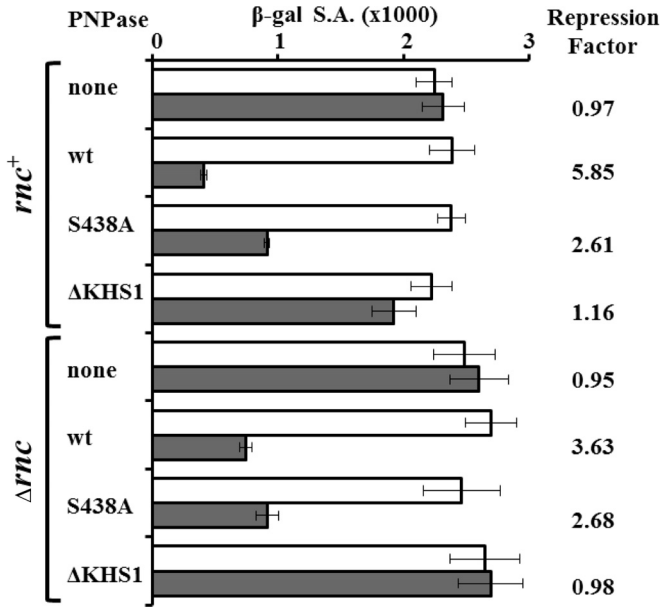

FIG 2 RNase III-independent PNPase autogenous regulation. β-Galactosidase activity expressed from *pnp-lacZ* translational fusion was determined. Cultures of strain C-5691(λGF2), a Δ*pnp-751* strain lysogenic for λ phage harboring a *pnp-lacZ* operon fusion, and its Δ*rnc-38*::*kan* derivative C-5979(λGF2) harboring pBAD24 (empty vector [none]), pBAD-Pnp$^+$ (wt), pBAD-pnpS438A, or pBAD-pnpΔKHS1 were grown in LD with either arabinose or glucose to induce or repress, respectively, transcription of the cloned *pnp* allele. Culture samples were assayed for β-galactosidase activity expressed from the *pnp-lacZ* translational fusion of prophage λGF2, as detailed in Materials and Methods. The histogram reports specific activity (S.A.) as nmol of 2-nitrophenyl-β-D-galactopyranoside converted to *o*-nitrophenol/min/mg of proteins. The repression factor is the ratio of β-galactosidase specific activity in cultures with uninduced (white bars) and induced (gray bars) PNPase.

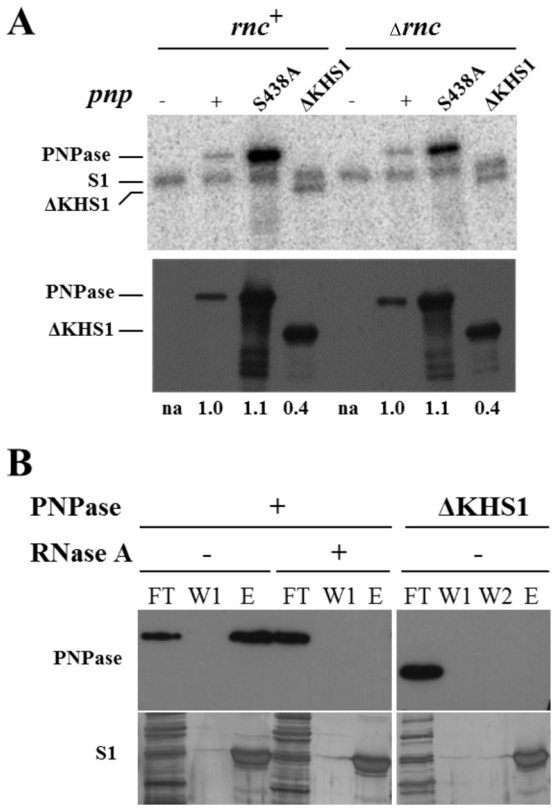

FIG 3 RNA binding by PNPase mutants. (A) PNPase-RNA UV cross-linking in crude cell extracts. Crude cell extracts (0.4 μg) of strains listed in the legend to Fig. 2 grown in LD arabinose to induce transcription of the cloned *pnp* allele were incubated with 100,000 cpm of uniformly ^{32}P-labeled PNPA247 RNA probe (1 nM), and the samples were UV irradiated (254 nm at 2.8 J cm^{-2}). The reaction products were then digested with RNase A, fractionated by 10% SDS-PAGE, and visualized by phosphorimaging (top) or Western blot analysis with anti-PNPase antibodies (bottom). The bands from the controls lacking PNPase correspond to S1 ribosomal protein (34). The values below the lanes refer to PNPase binding efficiency. The signal intensity of each sample was quantified by ImageQuant and normalized to the wild-type PNPase signal. Binding efficiency is given as the ratio of normalized cross-linking and Western blotting signals. na, not applicable. (B) S1-PNPase interaction. Crude extracts of strains expressing either wild-type PNPase (+) or the PnpΔKHS1 variant were incubated with S1-coated magnetic beads. Where indicated (RNase A +), the extracts were pretreated with RNase A to degrade bulk mRNA. After incubation of the beads with the extracts and washing, S1 (along with interacting proteins and RNA) was eluted as detailed in Materials and Methods. Proteins in different fractions were separated by SDS-PAGE and either blotted on a nitrocellulose filter and immunodecorated with PNPase-specific antiserum (top) or silver stained (bottom). Ten-microliter aliquots of each fraction were loaded on the gel for silver staining, whereas 1-μl (FT, W1, and W2) or 10-μl (E) amounts were analyzed by Western blotting. FT, crude extract after incubation with S1-coated beads; W1, first wash; W2, second wash; E, proteins coeluting with S1.

whereas Pnp-ΔKHS1 exhibited reduced RNA binding activity, as previously described (34).

Autogenous regulation by the *pnp*-ΔKHS1 mutant was severely impaired in both *rnc*$^+$ (as previously shown in references 31, 32, and 34) and Δ*rnc-38* strains (Fig. 2). In contrast, the *pnp*-S438A mutation only partially reduced repression in either strain. Namely, the repression factor was 2.61-fold in the *rnc*$^+$ strain (about the half of maximum repression attained by wild-type PNPase in the *rnc*$^+$ strain) and 2.68 in the Δ*rnc-38* background (about 74% of the wild-type PNPase repression in the same background).

Overall, these data suggest (i) that PNPase acts as a repressor of its own expression on the native (not processed by RNase III) *pnp* transcript and (ii) that PNPase phosphorolytic activity is dispensable for RTI regulation. Thus, this mechanism acts upstream from and in addition to the control of *pnp* mRNA stability exerted on RNase III-processed mRNA. Since it has been shown that, in the absence of RNase III, the stability of *pnp* mRNA is not affected by PNPase (15), such an RTI regulation of *pnp* operon expression should depend on translational repression by PNPase.

It should be mentioned that, although in our system, the genes encoding the wild-type and mutant PNPases cloned under the *araBp* promoter lack the 5′-UTR regulatory regions, the PNPase intracellular concentration in the arabinose-induced cultures was higher for the mutants than for the wild type, both in the *rnc*$^+$ and the Δ*rnc-38* strains (Fig. 3A). This could probably depend on the higher copy number of the ColE1-type vector expressing mutant PNPases (35). However, in spite of the higher level of Pnp-ΔKHS1, the RTI pathway was impaired in the strain complemented by *pnp*-ΔKHS1, thus further supporting the key role of PNPase RNA binding domains in the RTI mechanism.

PNPase and ribosomal protein S1 competitive binding to the *pnp* mRNA leader sequence. We previously showed by UV cross-linking experiments that both PNPase and ribosomal protein S1 bind to the *pnp* mRNA leader region RNA and modulate the sta-

bility of this messenger (28). It is thus conceivable that PNPase may prevent translation by competing with S1 (and/or the 30S ribosomal subunit) for binding to specific sites. Alternatively, PNPase could interact with S1 and form a complex that binds to *pnp* mRNA and interferes with its translation. The latter hypothesis, however, was not supported by the observation that, in an *E. coli* crude extract, S1-coated beads were able to capture wild-type PNPase but not the ΔKHS1 mutant enzyme (Fig. 3B). Moreover, if the extract was pretreated with RNase A to destroy RNA, wild-type PNPase-S1 interaction was no longer detectable (Fig. 3B); this suggests that S1 and PNPase are tethered by RNA rather than interacting directly with each other.

To test the former hypothesis, i.e., PNPase-S1 competition for the *pnp* mRNA leader region, we performed competitive RNA-protein cross-linking by adding increasing concentrations of PNPase to the PNPA157 RNA probe, which extends 157 nt from the *pnp-p2* promoter and covers the 5′-UTR and the translation initiation region (TIR) (Fig. 1), incubated with S1. As shown by the results in Fig. 4A, 9 to 12 nM PNPase is sufficient to displace S1 protein (30 nM; half saturation) from the RNA probe. Moreover, the affinity of the PNPA157 RNA probe for PNPase, as measured by EMSA, was much stronger than its affinity for S1 (dissociation constant [K_d] = 1.8 and 65 nM, respectively) (Fig. 4B). In addition, no bands other than those imputable to either PNPase or S1 could be detected, thus suggesting that the two proteins do not form heteromeric complexes on this RNA. We also measured the RNA affinity of PNPase and S1 by EMSA using different RNA probes covering different regions downstream from the *pnp-p2* promoter. The results presented in Fig. 4C show that PNPase in all cases exhibits higher affinity than S1; moreover, both PNPase and S1 show low affinity for probe PNPA101 (covering the region from +1 to +101, which covers the primary RNase III stem; K_d = 5 and 44 nM for PNPase and S1, respectively) and the highest affinity with probe PNPD146, which covers the region from +101 to +247, downstream from the RNase III stem (K_d = 1.6 and 22 nM, respectively) (Fig. 4C).

Overall, these data indicate that PNPase and ribosomal protein S1 can bind the 5′-UTR of *pnp* mRNA competitively and with differential affinities.

DISCUSSION

Both models for PNPase autogenous regulation proposed by Portier and collaborators (15, 16) posit a two-step mechanism: first, RNase III creates the substrate for PNPase (14), which in turn destabilizes the RNase III-processed *pnp* mRNA. In the former model, it was proposed that PNPase induces the degradation of its RNase III-processed messenger by preventing its translation (15). In the elegant later model (16), PNPase controls its own expression by degrading the small RNA, generated by RNase III cleavage, in the double-stranded structure that protects the 5′ end of the processed *pnp* mRNA UTR. Within this model, it was shown that degradation of the protective small RNA by PNPase directs the RNase III-processed *pnp* mRNA to an RNase E-dependent decay pathway and that PNPase was not implicated in translational repression of the stemless mRNA (17). On the other hand, some previously published data suggest a more complex scenario. For instance, point mutations mapping in the immediate proximity of the *pnp* Shine-Dalgarno region (and thus located more than 70 nt downstream from the RNase III cut site) affect *pnp* autoregulation by reducing the extent of repression by PNPase 2- to 3-fold (Table 2, strains GFX5311 and GFV5311) (14), a phenotype that is not easy to reconcile with the current model of *pnp* autoregulation.

In this work, we show that another mechanism is involved in PNPase autoregulation. We found that in the presence of ectopically expressed PNPase, the repression of a *pnp-lacZ* translational fusion in a Δ*rnc* strain is reduced to about half of that obtained in the *rnc*$^+$ background but not abolished. This clearly indicates that, in addition to the RNase III-dependent pathway (RTD), an RNase III-independent (RTI) pathway contributes to PNPase autogenous regulation. In agreement with an RTI pathway, in a different *E. coli* strain and with a similar reporter system, Robert-Le Meur and Portier (14) found a 2-fold repression by PNPase expressed at the *pnp* chromosomal locus in a Δ*rnc* background [see reference 14, Table IV, column headed "pBPΔ7 (*rpsO*)," ratio for the strains described as GF494 (*rnc*$^-$ *pnp*$^-$) and GF493 (*rnc*$^-$ *pnp*$^+$)]. In contrast, however, essentially no repression by PNPase expressed from a plasmid was found in the strain described as GF494 (*rnc*$^-$ *pnp*$^-$) (see reference 14, Table IV, GF494 repression ratio of 1.1). This discrepancy was not addressed.

Additional evidence for a PNPase-dependent, RTI pathway is provided by the observation that a PNPase mutant lacking phosphorolytic activity but proficient in RNA binding partially regulates *pnp-lacZ* expression both in the *rnc*$^+$ and in the Δ*rnc* background with similar efficiency, whereas, consistent with previous data (32, 36, 37), PNPase mutants with defects in the RNA binding domains could not. Thus, the RTI pathway acts via RNA binding. Since the native *pnp* mRNA (not processed by RNase III) is also very stable in the presence of PNPase and is intrinsically translatable (13, 17, 38), we suggest that PNPase binding prevents its translation. This could reconcile evidence for translational repression participating in PNPase autogenous regulation (14, 15) with the observation that PNPase is not implicated in translational repression in the RTD pathway (17).

Point mutations or deletions of the RNA binding domains KH and/or S1 affect substrate affinity (31, 33, 34, 39). Interestingly, a strong correlation between RNA affinity and autoregulation has been observed (32). These data may fit the RTD model by implying that PNPase recruitment to the *pnp* mRNA UTR is a limiting step in autoregulation. In addition, mutations in the RNA binding domains may also, to different extents, reduce PNPase catalytic activity (31, 33, 34, 39), and this could reduce the efficiency of degradation of the protecting small RNA. However, the RTI model provides an additional, although not mutually exclusive, mechanism that may contribute to the above-described correlation, as mutations impairing RNA binding are predicted to affect translational repression. It thus appears that PNPase interaction with the 5′-UTR of its mRNA may have a dual effect, as follows. (i) Before RNase III processing, PNPase binding inhibits translation. The RTI pathway is reversible, since the unprocessed *pnp* primary transcript is also very stable in the presence of PNPase (38). (ii) Upon RNase III processing, PNPase activity degrades the small protective RNA and irreversibly directs the stemless mRNA to the RNase E-dependent degradation pathway.

The primary *pnp* transcript has been shown to be translatable, whereas the mature mRNA, not annealed with the protecting small RNA, appears not to be (13, 17, 38). Our data suggest that the RNase III-processed mRNA annealed with the small RNA, before PNPase degrades it, is also translatable. In fact, if translation from the *pnp* 5′-UTR would only occur before RNase III cleavage, the downstream RTD step, although relevant for the

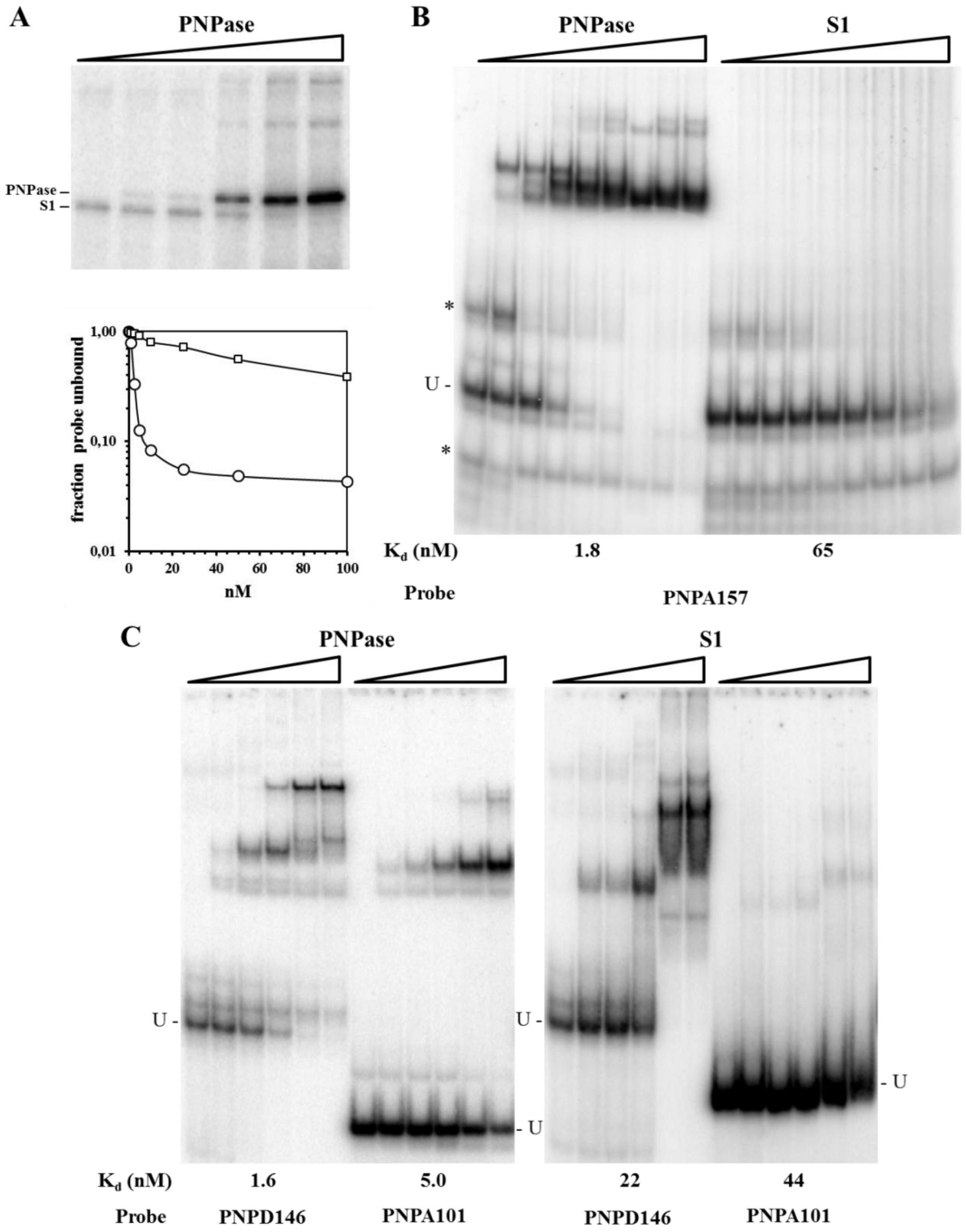

FIG 4 PNPase and S1 binding to the *pnp* mRNA leader region. (A) PNPase-S1 competitive cross-linking for *pnp* mRNA leader. Uniformly labeled (100,000 cpm) PNPA157 probe (0.6 nM) was incubated for 20 min at 21°C with 30 nM S1 and increasing (0, 1, 3, 6, 9, and 12 nM) concentrations of PNPase. The samples were then UV irradiated (254 nm at 2.8 J cm^{-2}), digested with RNase A, and fractionated by SDS-PAGE. (B and C) PNPase and S1 binding to different regions of *pnp* mRNA leader. EMSA was performed as described in Materials and Methods, using 5′-end-labeled [^{32}P]PNPA101 (14,000 cpm, 0.5 nM) or [^{32}P]PNPD146 (30,000 cpm, 0.5 nM) probes incubated for 20 min at 21°C with increasing concentrations of PNPase (0, 0.5, 1, 2, 4, and 8 nM) or S1 (0, 2, 4, 12, 36, and 72 nM). The unbound probe band intensities were evaluated by ImageQuant, normalized to the intensity of the 0 nM PNPase or S1 sample, and plotted versus the PNPase or S1 concentration; the dissociation constant (K_d) was evaluated as the PNPase or S1 concentration giving 50% probe binding. A plot is shown as an example on the left of the image in panel B. K_d is indicated at the bottom of each panel. U, unbound probe; *, signals likely due to alternative conformations of the unbound probe.

control of *pnp* mRNA stability (13, 14), would nevertheless be uninfluential in PNPase expression. Our data, on the contrary, show stronger repression levels when the RTI and RTD steps are both operating than when only the RTI pathway is operating (Fig. 2, compare results for [i] Pnp^+ and Pnp-S438A in the rnc^+ background and [ii] Pnp^+ in the rnc^+ and Δ*rnc-38* backgrounds). This supports the idea that, in the RTD pathway, PNPase may act by converting a translatable form of *pnp* mRNA (with an RNase III-truncated stem at the 5′ end) into an untranslatable stemless molecule, which would be quickly degraded through an RNase E-dependent decay pathway.

We previously showed that PNPase and the ribosomal protein S1 are the two main proteins able to bind the 5′-UTR of *pnp* mRNA (28). We thus suggest that inhibition of *pnp* mRNA translation in the RTI pathway occurs via PNPase competition with S1 for RNA binding. Consistent with this hypothesis, we observed *in vitro* that PNPase competes with and completely displaces S1 from the 5′-UTR of *pnp* mRNA at a 3-fold-lower concentration than the ribosomal protein (Fig. 4A). In agreement with this observation, PNPase exhibits a much higher affinity than S1 for the 5′-UTR of *pnp* mRNA (Fig. 4B and C). This higher affinity may allow the PNPase to compete with the much more abundant S1 protein (40) for the interaction with the *pnp* mRNA.

In conclusion, we have identified a novel, PNPase-dependent and RNase III-independent pathway that contributes to PNPase autogenous regulation in *E. coli*. This RTI pathway, unlike RTD autoregulation, is reversible and does not require the catalytic activity of the enzyme, as PNPase binds to the native *pnp* mRNA and likely prevents its translation. PNPase thus plays a direct role in its autogenous regulation before the primary transcripts become engaged in the downstream RTD branch.

As the activity of RNase III is downregulated in response to different stresses (41), it is possible that the relative effects of the two PNPase autoregulation pathways change under different physiological conditions. The two sides of the autogenous regulation process highlight the interplay between translation and RNA decay machineries in fine-tuning the expression of a pleiotropic gene.

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