Recycling of a regulatory protein by degradation of the RNA to which it binds

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When *Bacillus subtilis* is grown in the presence of excess tryptophan, transcription of the *trp* operon is regulated by binding of tryptophan-activated TRAP to *trp* leader RNA, which promotes transcription termination in the *trp* leader region. Transcriptome analysis of a *B. subtilis* strain lacking polynucleotide phosphorylase (PNPase; a 3′-to-5′ exoribonuclease) revealed a striking overexpression of *trp* operon structural genes when the strain was grown in the presence of abundant tryptophan. Analysis of *trp* leader RNA in the PNPase$^-$ strain showed accumulation of a stable, TRAP-protected fragment of *trp* leader RNA. Loss of *trp* operon transcriptional regulation in the PNPase$^-$ strain was due to the inability of ribonucleases other than PNPase to degrade TRAP-bound leader RNA, resulting in the sequestration of limiting TRAP. Thus, in the case of the *B. subtilis trp* operon, specific ribonuclease degradation of RNA in an RNA–protein complex is required for recycling of an RNA-binding protein. Such a mechanism may be relevant to other systems in which limiting concentrations of an RNA-binding protein must keep pace with ongoing transcription.

Transcription attenuation is a form of gene regulation in which transcription of a gene or operon is regulated by the folding of a leader RNA to form either a transcription terminator, thus preventing transcription of downstream structural genes, or an antiterminator, thus allowing transcription to proceed (1, 2). Regulated expression of structural genes in the *trp* operon of several bacteria has been fertile ground for elucidating various transcription attenuation mechanisms. In *Escherichia coli*, the *trp* leader terminator structure is formed when tryptophan is abundant, and the antiterminator structure forms when tryptophan is scarce, because of ribosome stalling at tryptophan codons in the leader peptide coding sequence. In *Lactococcus lactis*, it is likely that formation of the antiterminator structure depends on binding of uncharged $tRNA^{Trp}$ to leader RNA (the "T-box" mechanism) (1, 2). In both of these cases, the terminator structure is the default; the antiterminator structure, which allows transcription of *trp* structural genes, is formed only in response to a scarcity of tryptophan. By contrast, in *Bacillus subtilis* the *trp* leader antiterminator structure is the default. Formation of the terminator structure depends on binding of [1]TRAP, a regulatory protein that can bind *trp* leader RNA only when it is activated by tryptophan (3–5). In conditions of low tryptophan, TRAP does not bind *trp* leader RNA, allowing formation of the antiterminator structure and transcription of the *trp* operon structural genes. When tryptophan is abundant, a TRAP 11-mer is activated, which can then bind to 11 trinucleotide repeats in the *trp* leader RNA, resulting in the formation of the terminator structure (6, 7).

There is a long-standing question regarding TRAP-mediated regulation of the *B. subtilis trp* operon: How can a limited amount of TRAP suffice to keep pace with ongoing transcription from the *trp* promoter? There is no evidence for regulation of transcription initiation at the *trp* promoter (3); thus, *trp* leader RNA is synthesized constitutively. On the other hand, the steady state level of TRAP is ≈300 complexes per cell when grown in minimal medium (P. Gollnick, personal communication). To assure continued termination of transcription in the presence of abundant tryptophan, TRAP must bind to the nascent *trp* leader transcript before RNA polymerase transcribes past the terminator. Although NusA-stimulated RNA polymerase pausing provides additional time for TRAP binding (8), it appears that efficient transcription termination would require an available excess of TRAP. *B. subtilis* does have an anti-TRAP protein (AT), which appears to inhibit binding of TRAP to *trp* leader RNA by masking TRAP RNA-binding sites (9, 10). However, there is no evidence that AT is able to remove TRAP from *trp* leader RNA once TRAP is already bound. How then is TRAP released from terminated *trp* leader RNA to ensure continuing transcription termination? Here, we describe experiments indicating that degradation of *trp* leader RNA by the 3′-to-5′ exoribonuclease polynucleotide phosphorylase (PNPase) is required for efficient release of TRAP. Other 3′-to-5′ exoribonucleases of *B. subtilis* cannot substitute for PNPase in this role.

Materials and Methods

Bacterial Strains. The *B. subtilis pnpA*$^+$ strain was BG1, which is *trpC2 thr-5*. The *pnpA* mutant was BG119, a derivative of BG1 in which an internal portion of the *pnpA* gene has been replaced with a kanamycin resistance gene (11). Some of the control experiments for TRAP-specific binding in the protein extract were done with BG4233, an *mtrB* deletion mutant (12).

RNA Isolation and Two-Channel *B. subtilis* cDNA Microarray Experiments. *B. subtilis* strains were grown to mid-exponential phase in minimal medium containing Spizizen salts with 0.5% glucose, 0.1% casamino acids, 0.001% yeast extract, 0.1% tryptophan and threonine, and 1 mM $MgSO_4$. Total RNA was extracted essentially as described (13), except that the buffer for phenol extraction (ANE buffer) was replaced with 50 mM sodium acetate/1 mM EDTA (pH 6.0). Procedures for labeling with cyanine 3-dCTP (Cy3) and cyanine 5-dCTP (Cy5) dyes (Perkin–Elmer) were exactly as described (14). The Cy3-cDNA (from the reference strain BG1) and Cy5-cDNA (from BG119) probes were concentrated on an Amicon 30 column (Millipore) to a 5-μl volume and mixed. Twenty-five microliters of 10 mg/ml herring sperm DNA was added, and the volume was adjusted with H_2O to 100 μl. The mixture was heated for 5 min at 95°C and mixed with an equal volume of hybridization buffer [final concentration: 5× SSC/25% formamide/0.2% SDS/0.2 μg/ml BSA (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7)]. The mixture was hybridized to *B. subtilis* microarray slides (Eurogentec, Brussels) overnight at 42°C in an Amersham Pharmacia Lucidea SlidePro hybridization station. The microarray slides contained whole ORF sequences, and each *B. subtilis* gene was represented twice on the microarray. The slides were washed with 0.1× SSC and 0.2% SDS, dried, and scanned at excitation wavelengths 550 nm (Cy3) and 640 nm (Cy5) in a ScanArray Express HT scanner (Perkin–Elmer). Fluorescence

Abbreviations: PNPase, polynucleotide phosphorylase; TRAP, *trp* RNA-binding attenuation protein.

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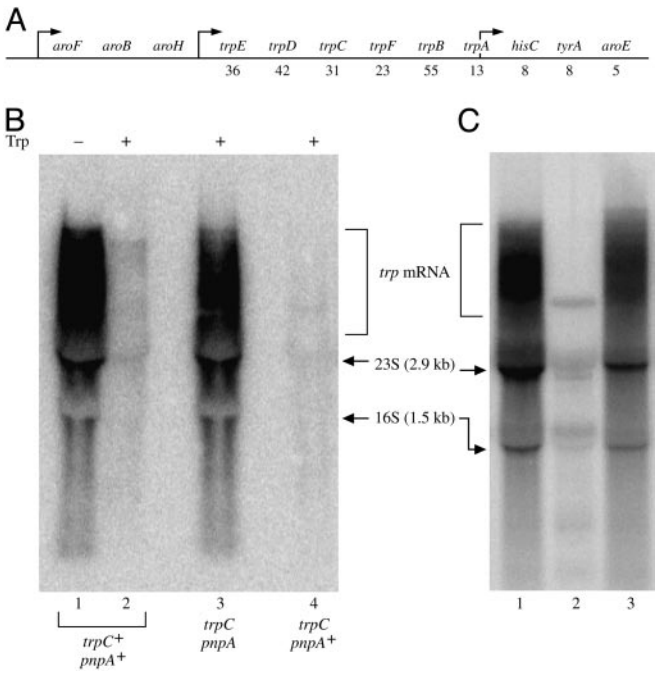

Fig. 1. (*A*) Fold overexpression of *trp* operon genes from microarray experiments. The order of genes in the aromatic amino acid supraoperon is depicted. Promoters are indicated by hooked arrows. Numbers below genes indicate level of expression in the PNPase$^-$ strain relative to wild type. Values are averages of four determinations, two measurements each from two independent RNA isolations. (*B*) Northern blot analysis of *trp* operon mRNA in wild-type and *pnpA* strains. Total RNA was separated on a formaldehyde-agarose gel and probed with a uniformly labeled *trpA* antisense RNA. Migration of 23S and 16S ribosomal RNAs is indicated. Strains were grown in the presence (+) or absence (−) of tryptophan. (*C*) Northern blot analysis of *trp* operon mRNA in the *pnpA* mutant strain, carrying no plasmid (lane 1), vector plasmid + *mtrB* gene (lane 2), and vector plasmid alone (lane 3). The probe for both Northern blots shown was an antisense *trpA* probe.

was measured at 570 nm (Cy3) and 670 nm (Cy5). Hybridization, washing, and scanning were performed at the Mount Sinai Microarray Shared Research Facility.

The fluorescence intensity of microarray spots was quantified and analyzed by using the SCANARRAY EXPRESS program. The Lowess method (15) was used for normalization of raw fluorescence intensity. Only spots with mean fluorescence intensity five times higher than background were analyzed. A normalized Cy5/Cy3 mean fluorescence ratio was used to quantify changes in gene expression. Genes with Cy5/Cy3 mean fluorescence ratio >3 were considered significantly up-regulated, whereas genes with mean fluorescence ratio <0.33 were considered significantly down-regulated. Preliminary control experiments with cDNA synthesized on BG1 RNA and labeled with Cy3 and Cy5 showed a ratio of 0.5–2.0 for 90% of genes on the microarray. A normalized Cy5/Cy3 mean fluorescence ratio >3 or <0.33 was not detected for any gene in control experiments. Quantitative microarray data (Fig. 1*A*) are the mean of results from four spots on the microarray, obtained from two independent RNA isolations.

Northern Blot Analysis. RNA isolation was as described above for microarray experiments. Total cellular RNA was separated in 6% denaturing polyacrylamide or 1% agarose-formaldehyde gels and analyzed as described (16). Electroblotting from 6% high-resolution gels was done as described (17). Uniformly labeled, antisense riboprobes were synthesized by T7 RNA polymerase transcription, in the presence of [^{32}P]UTP, of gel-purified DNA fragments that were generated by PCR amplification of the *trp* leader sequence (nucleotides 1–132 of the *trp* operon) or *trpA* and *trpD* coding sequences. The upstream primer in the PCRs contained the 17-nt T7 RNA polymerase promoter sequence at its 5′ end.

Analysis of *trp* Leader RNA Degradation *in Vitro*. Protein extracts were prepared from BG1 as described (18), except that cells were disrupted by sonication and the final supernatant was dialyzed overnight against 200 volumes of 20 mM Tris·HCl (pH 7.75)/100 mM KCl/10% glycerol/0.2 mM EDTA/1 mM DTT/1 mM phenylmethylsulfonyl fluoride. Dialysis was repeated for 1 h in fresh buffer, and the dialysate was stored at −70°C.

Five micrograms of protein extract were used for each reaction. *trp* leader RNA was synthesized by T7 RNA polymerase transcription, as described above for riboprobes. The *trp* leader RNA contained nucleotides 1–111 of the native *trp* operon sequence, with three guanosine residues added at the 5′ end to achieve efficient T7 RNA polymerase transcription. Two to 5 pmol of gel-purified *trp* leader RNA were 5′-end-labeled with [^{32}P]ATP by using T4 polynucleotide kinase. For each reaction, 0.02 pmol of labeled RNA was used in a buffer that contained 50 mM Na-tricine (pH 8.0), 100 mM KCl, and 1 mM DTT. Additions were 1.25 mM tryptophan and 1 mM $MnCl_2$, with or without 1 mM Na_2HPO_4 (pH 7.5). Reactions were incubated at 37°C for 35 min, followed by phenol:chloroform (1:1) extraction and ethanol precipitation of the aqueous phase. Precipitated RNA was resuspended in 10 μl of diluted RNA loading dye (Ambion, Austin, TX), and 5 μl was run on either 6% low-resolution or high-resolution 8 M urea denaturing polyacrylamide gels.

Gel mobility-shift analysis of TRAP binding was done as described (19), but in the buffer used to assay degradation of *trp* leader RNA.

Results and Discussion

***B. subtilis* Transcriptome Analysis Reveals Overexpression of *trp* mRNA in a PNPase Mutant.** Disruption of the *B. subtilis pnpA* gene, encoding PNPase, results in a number of phenotypes, including cold sensitivity, reduced competence, filamentous growth, tetracycline sensitivity, and accumulation of mRNA decay intermediates (11, 20, 21). In an attempt to understand the effect of eliminating PNPase activity on mRNA levels, microarray experiments were performed in which the expression of 96% of all *B. subtilis* ORFs (3,925) was compared in a wild-type vs. a PNPase-disrupted strain. The PNPase-disrupted strain and the "wild-type" reference strain from which it was derived are tryptophan auxotrophs (11), and so must be grown in the presence of tryptophan. Tryptophan auxotrophy in these strains is due to the *trpC2* mutation, which is a 3-bp in-frame deletion in the *trpC* coding region (22). Thus, any effects on *trp* operon transcription could not be attributed to this mutation.

An unexpected finding of the transcriptome analysis was that *trp* operon genes were highly overexpressed in the *pnpA* mutant strain (Fig. 1*A*). In addition to the *trp* operon structural genes, the downstream *hisC*, *tyrA*, and *aroE* genes were also significantly overexpressed in the *pnpA* mutant. Three other genes (*pabA*, *trpP* [*yhaG*], and *ycbK*) are regulated by TRAP, but at the level of translation (3). Of these, only *pabA* showed a small increase in expression (4-fold), which was just above the cutoff of 3-fold for significant up-regulation.

The microarray result was confirmed by Northern blot analysis, using a *trpA* anti-sense RNA probe (Fig. 1*B*; similar results were obtained with a *trpD* probe). In a wild-type strain (i.e., *trpC*$^+$ *pnpA*$^+$), *trp* operon mRNA ranging from ≈4 kb to 9 kb was detected when the strain was grown in the absence of tryptophan (Fig. 1*B*, lane 1). Presumably, the smear of *trp*-specific RNA represented degradation or premature termination products of

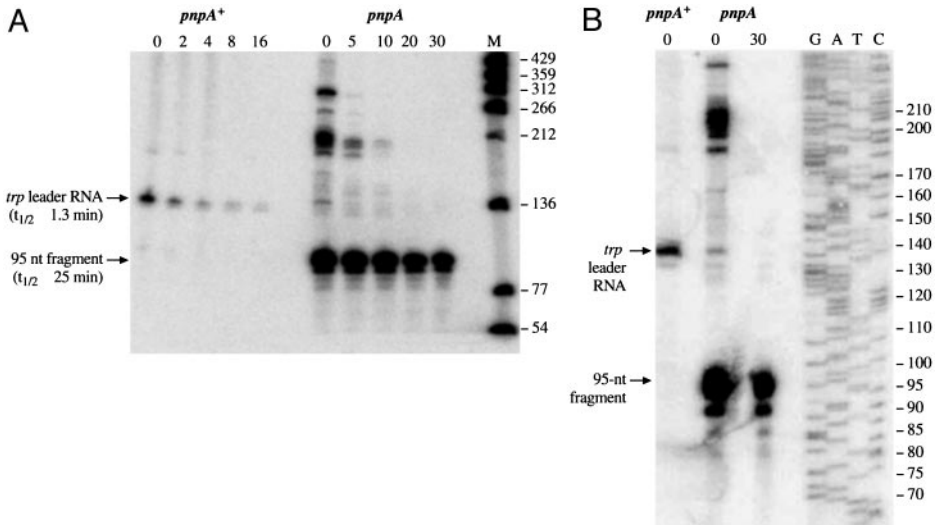

Fig. 2. Northern blot analysis of *trp* leader RNA. (*A*) Total RNA was isolated at various times after addition of rifampicin to inhibit new transcription. Above each lane is the time (minutes) after rifampicin addition. RNA was separated in a denaturing 6% polyacrylamide gel and probed with *trp* leader antisense RNA. Migration of full-length *trp* leader RNA and a protected fragment of *trp* leader RNA are indicated on the left. Sizes of 5′-end-labeled DNA fragments (in base pairs) are indicated to the right of the marker lane (M). (*B*) High-resolution separation of total RNA, followed by probing with *trp* leader antisense RNA. A DNA sequencing ladder was run in parallel as a size marker.

transcripts initiating from the *trp* promoter and the upstream *aro* promoter. The *trp* operon signal was barely detectable when the wild-type strain was grown in the presence of tryptophan (Fig. 1*B*, lane 2). This was due to tryptophan-activated TRAP binding to *trp* leader RNA, which results in transcription termination in the *trp* leader region. Similarly, when a *trpC* strain that was wild-type for PNPase was grown in the presence of tryptophan, the signal was barely detectable (Fig. 1*B*, lane 4). However, in the *trpC pnpA* strain, intense bands representing *trp* mRNA were detected, even though the strain was grown in the presence of tryptophan (Fig. 1*B*, lane 3). The pattern in the *trpC pnpA* strain grown in the presence of tryptophan was similar to that of the wild-type grown in the absence of tryptophan (compare lanes 1 and 3). An analysis of *trp* operon mRNA stability showed no difference between the wild-type and *pnpA* strains (data not shown). Thus, the large increase in *trp* mRNA in the *pnpA* strain was not due to mRNA stabilization but to a failure to terminate transcription in the *trp* leader region when tryptophan was abundant.

A Hypothesis to Explain *trp* Overexpression in the *pnpA* Mutant. Our hypothesis to explain these results was as follows. In the wild-type strain, the presence of tryptophan allows TRAP to bind tightly to *trp* leader RNA. PNPase is responsible for degrading *trp* leader RNA that is bound by TRAP, thus releasing TRAP for binding of newly synthesized *trp* leader RNA. In the absence of PNPase, the remaining 3′-to-5′ exoribonucleases are unable to compete with TRAP for access to *trp* leader RNA. Thus, *trp* leader RNA is stabilized and the limiting amount of TRAP becomes sequestered on the leader RNA. The concentration of free TRAP drops below what is needed to prevent new *trp* transcription from reading into the structural genes, resulting in a large increase in the steady-state level of *trp* operon mRNA. According to this hypothesis, an accumulation of TRAP-protected *trp* leader RNA should be observed in the PNPase mutant strain.

Northern blot analysis was performed to examine the decay of *trp* leader RNA (Fig. 2*A*). In the wild-type strain, where PNPase was present, a 140-nt leader RNA was detected, and this RNA decayed with a short half-life (1.3 min). This RNA had the expected size for a transcript that results from termination at the *trp* leader attenuator. However, in the PNPase-disrupted strain, a smaller *trp* leader RNA fragment of ≈95 nt was detected. The TRAP binding target extends from nucleotides 36–91 (6); thus, the 95-nt RNA had the expected size for a TRAP-protected RNA fragment. This 95-nt RNA was 15 times more abundant than the steady-state level in the wild-type strain (time 0 after rifampicin addition) and had an extremely long half-life (25 min). In addition, a faint ladder of bands below this major band was detected. In the PNPase mutant, some full-length 140-nt leader RNA was also detected, as well as higher molecular weight RNA of ≈200 nt and a distinct band of ≈280 nt. We believe that the RNAs around 200 nt in size represent blocks to 3′-to-5′ exoribonuclease degradation near the base of the TRAP-dependent RNA structure that sequesters the *trpE* Shine–Dalgarno sequence (23, 24). The 280-nt band may be the result of a block to exonucleolytic decay by a relatively strong stem–loop structure (−8.2 kcal/mol) that is predicted to form within the *trpE* coding region.

To determine more precisely the size of the small *trp* leader RNA fragments, Northern blot analysis was performed by using a high-resolution polyacrylamide gel, with a DNA sequence ladder run in parallel (Fig. 2*B*). A *trp* leader RNA of the expected length (140-nt) was observed in the wild-type strain. In the *pnpA* mutant strain, the observed *trp* leader RNA fragment migrated as an intense band, or a set of bands, of ≈95 nt, and bands with decreasing intensity could be detected migrating at 5-nt intervals below the major band. Most likely, this pattern represented aborted attempts by a 3′-to-5′ exoribonuclease other than PNPase to degrade *trp* leader RNA. TRAP binding depends on 11 (G/U)AG repeat sequences that are separated by two or three nonconserved spacer residues (6). According to our hypothesis, PNPase competes effectively against TRAP for *trp* leader RNA binding and rapidly degrades the leader RNA. Other exonucleases may do so poorly, being able to compete with TRAP to some extent only at the most distal GAG triplet repeat, at which TRAP binds least tightly (6). Because the last four repeats are all separated by 2-nt spacers, the 5-nt ladder that became fainter with increasing distance from the 3′ edge of the TRAP binding target was likely due to sequential degradation of 5-nt segments containing one triplet repeat plus a 2-nt spacer.

The results from Northern blot analyses were essentially confirmed by ribonuclease protection assay (data not shown).

Experiments *in Vitro* to Confirm the Role of PNPase in *trp* Leader RNA Decay. A dialyzed protein extract from a *pnpA*$^+$ strain was used to observe PNPase-dependent decay of the *trp* leader RNA, as well as TRAP-dependent protection of *trp* leader RNA in the absence of PNPase activity. PNPase is a phosphorolytic enzyme; thus, in the dialyzed extract, PNPase would be inactive unless inorganic phosphate (P_i) were added. A 114-nt, 5′-end-labeled *trp* leader RNA was prepared that contained three guanosine residues at the 5′-end (for efficient T7 RNA polymerase transcription), followed by the first 111 nt of the *trp* leader RNA sequence. In control experiments (not shown), binding of purified TRAP, or TRAP in a BG1 cell extract, to the 114-nt *trp* leader RNA was examined in gel mobility-shift experiments, as described (19). TRAP binding was shown to be completely dependent on the addition of tryptophan. Furthermore, control experiments with extracts from an *mtrB* strain (i.e., that contained no TRAP) showed no tryptophan-dependent protein binding to the 114-nt RNA. Therefore, addition of tryptophan to the extract would result in specific binding of TRAP to the 114-nt RNA.

Analysis of decay of the 114-nt RNA in the extract is shown in Fig. 3*A Left*. In the absence of tryptophan, the RNA was degraded appreciably in the presence of Mn^{2+} (lane 2) and completely in the presence of Mn^{2+} + P_i (lane 3). In experiments not shown, decay of the 114-nt RNA was not observed when Mg^{2+} rather than Mn^{2+} was added. This was consistent with earlier observations that exonuclease activities in *B. subtilis* extracts, other than PNPase, were Mn^{2+}-dependent (11, 25). In the presence of tryptophan, an intermediate in the degradation of the 114-nt RNA was observed when Mn^{2+} was added (lane 5), corresponding to the 95-nt fragment observed *in vivo*. When P_i was added in addition to Mn^{2+} and tryptophan, the 114-nt RNA was completely degraded and no intermediate was observed (lane 6). A faint decay intermediate was also detected in the absence of added tryptophan (lane 2). This finding may be the result of trace amounts of tryptophan present in the extract (perhaps generated by proteolysis) that would activate TRAP for binding.

The results of the *in vitro* experiment indicated that PNPase activity was required for degradation of the *trp* leader RNA that was bound by TRAP. To rule out the possibility of another P_i-dependent ribonuclease activity, the *in vitro* experiment was done with an extract from a *pnpA* strain (Fig. 3*A Right*). In this case, when tryptophan was not present, a similar small amount of *trp* leader RNA decay was observed, whether or not P_i was added (lanes 2 and 3). Moreover, in the presence of tryptophan (i.e., when TRAP was bound), the same RNA decay intermediate was observed whether or not P_i was added (lanes 5 and 6). Comparing lanes 3 and 6 in Figs. 3*A Left* with lanes 3 and 6 in Fig. 3*A Right* showed clearly that PNPase was responsible for *trp* leader RNA decay.

To resolve the *in vitro*-generated RNAs, the same reaction products were separated on a high-resolution polyacrylamide gel (Fig. 3*B*). Intense bands in the low-resolution gel (Fig. 3*A*) were resolved in the high-resolution gel, indicating that the TRAP-protected RNA was actually a series of closely spaced RNAs. This pattern was consistent with aborted attempts by a 3′-to-5′ exonuclease to degrade past a TRAP-protected region. Thus, it is apparent that exoribonuclease(s) other than PNPase are blocked 2–6 nt downstream from what is known to be the edge of TRAP-bound RNA (6, 26). The size of the protected fragments in the *in vitro* assay (98–102 nt, not counting the three guanosine residues added at the 5′ end) was larger than what was determined from the Northern blot analysis in Fig. 2*B* (≈94–99 nt). This finding may reflect slight differences in the accessibility of *trp* leader RNA to ribonuclease activity *in vitro* vs. *in vivo*.

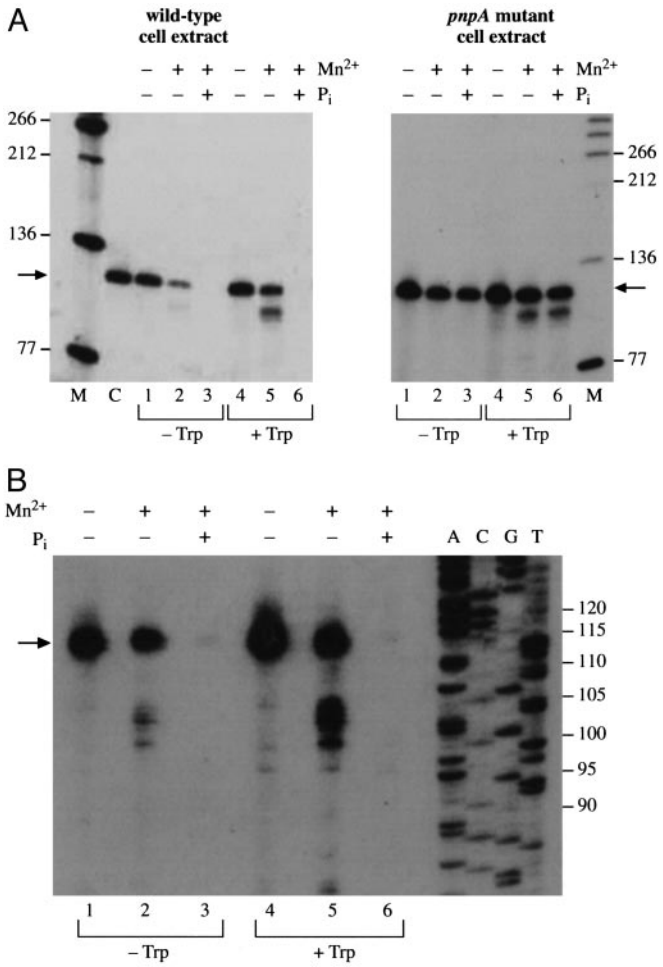

Fig. 3. Analysis of 114-nt *trp* leader RNA in *B. subtilis* protein extracts. (*A*) Low-resolution polyacrylamide gels. Size marker DNA fragments were in lane M. Control (lane C) contained substrate RNA that was not incubated in the extract. Substrate RNA (migration indicated by arrows) was incubated in the extract without (lanes 1–3) or with (lanes 4–6) addition of 1.25 mM tryptophan. Additions were none (lanes 1 and 4), 1 mM Mn^{2+} (lanes 2 and 5), or 1 mM Mn^{2+} + 1 mM NaH_2PO_4 (lanes 3 and 6). Results using wild-type cell extract (*Left*) and *pnpA* mutant cell extract (*Right*) are shown. (*B*) High-resolution polyacrylamide gel. Samples loaded in lanes 1–6 are the same as those in lanes 1–6 in *A Left*. Migration of substrate RNA is indicated by the arrow at the left. A DNA sequencing ladder is at the right, with sizes (in nt) indicated.

A Test of the Requirement for Available Free TRAP in Regulating *trp* Operon Transcription. Our model to account for the overexpression of *trp* mRNA in a PNPase$^-$ mutant is that recycling of a limiting amount of TRAP complex, by PNPase degradation of leader RNA, is required to provide enough free TRAP complex to keep pace with ongoing *trp* operon transcription. According to this model, supplying TRAP in much greater amounts should obviate the need for PNPase degradation of *trp* leader RNA. This was tested by transforming the PNPase$^-$ strain with plasmid pSI45, a multicopy plasmid that carries the *mtrB* gene (which encodes TRAP protein) expressed from its native promoter (27). Transcription of *trp* operon genes was examined in a Northern blot analysis (Fig. 1*C*) of RNA isolated from the following three strains grown in the presence of tryptophan (the strains are *trpC2*): *pnpA* mutant (lane 1), *pnpA* mutant with pSI45 (lane 2), and *pnpA* mutant with vector plasmid (lane 3).

These results demonstrate that the presence of multiple copies of the *mtrB* gene represses *trp* transcription, even in the absence of PNPase. Whereas the *pnpA* strain with a single *mtrB* gene in the chromosome showed an abundance of *trp* transcripts (lanes 1 and 3), the *pnpA* strain containing pSI45 showed only low levels of *trp* transcription. Thus, an excess of TRAP results in ongoing transcription termination and does not require PNPase-dependent recycling of the TRAP bound to *trp* leader RNA.

Conclusions. The results of *in vivo* and *in vitro* experiments demonstrate that regulation of the *B. subtilis trp* operon by transcription attenuation depends on PNPase degradation of terminated *trp* leader RNA. In the absence of PNPase, a stable *trp* leader RNA fragment was observed, the 3′ end of which corresponded roughly to the 3′ end of the TRAP-binding site. The TRAP complex binds *trp* leader RNA with high affinity (K_d = 0.12 nM; ref. 28). Binding of TRAP to *trp* leader RNA must occur after a sufficient number of triplet repeats have been transcribed but before RNA polymerase transcribes past the downstream nucleotides that form the transcription terminator structure. Thus, TRAP binding to newly synthesized RNA must be sufficiently rapid to achieve the desired transcription termination (8). However, this very same strong binding would lead to loss of regulation if TRAP could not be efficiently recycled. In *B. subtilis*, only the 3′-to-5′ exonucleolytic activity of PNPase is capable of degrading *trp* leader RNA that is bound by TRAP. Although other ribonucleases are able to compensate for the loss of PNPase in terms of overall mRNA decay (11), particular mRNA decay intermediates do accumulate in the absence of PNPase, and it has been proposed that the nature of the 3′ ends of these intermediates make them susceptible to PNPase degradation but not to degradation by other exoribonucleases (17). The ability of PNPase and not other ribonucleases to degrade TRAP-bound *trp* leader RNA may not be a measure of the strength of PNPase processivity but may reflect a unique way in which PNPase achieves RNA binding. A kinetic analysis using purified PNPase (which is not yet available) and other *B. subtilis* exoribonucleases will be needed to elucidate the specificity of TRAP-bound *trp* leader RNA decay.

In our microarray experiments, the only other genes that were overexpressed at levels similar to that of the *trp* operon were those of the proline uptake and utilization genes in the *ycg* operon: *ycgM* (38-fold), *ycgN* (30-fold), and *ycgO* (11-fold). It would be remarkable if such overexpression in the PNPase$^-$ strain would be predictive of a regulatory mechanism for *ycg* operon genes similar to that of *trp* operon genes. However, studies on *ycg* operon transcription have revealed that there is only a short (40 nt) untranslated leader sequence (S. Moses and E. Bremer, unpublished results). Computer-assisted analysis of the *ycg* operon 5′ sequence suggests that the *ycg* operon is unlikely to be regulated by a transcription attenuation mechanism.

Recycling of proteins or protein complexes involved in RNA function has been shown to be important in other systems, e.g., termination of translation (29), mRNA splicing (30), and mRNA transport (31). We believe that release of TRAP is a distinct form of recycling in that it depends on RNA degradation. We expect that our results can be generalized to other RNA-binding proteins that are in limiting concentrations and are involved in regulation of transcription, translation, or other posttranscriptional processes. Recycling of these proteins for ongoing function would depend on degradation of the RNAs to which they are bound. It is likely that, depending on the intrinsic strength of the RNA–protein complex, specific ribonucleases will be required to disrupt such complexes.

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