Analysis of lysine recognition and specificity of the Bacillus subtilis L box riboswitch

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ABSTRACT

The ever-changing environment of a bacterial cell requires sophisticated mechanisms to adjust gene expression in response to changes in nutrient availability. L box riboswitch RNAs regulate gene expression in response to cellular lysine (lys) concentrations in the absence of additional regulatory factors. In Bacillus subtilis, binding of lysine (lys) to the L box RNA causes premature transcription termination in the leader region upstream of the lysC coding sequence. To date, little is known about the specific RNA–lys interactions required for transcription termination. In this study, we characterize features of the B. subtilis lysC leader RNA responsible for lys specificity, and structural elements of the lys molecule required for recognition. The wild-type lysC leader RNA can recognize and discriminate between lys and lys analogs. We identified leader RNA variants with mutations in the lys-binding pocket that exhibit changes in the specificity of ligand recognition. These data demonstrate that lysC leader RNA specificity is the result of recognition of ligand features through a series of distinct interactions between lys and nucleotides that comprise the lys-binding pocket, and provide insight into the molecular mechanisms employed by L box riboswitch RNAs to bind and recognize lys.

INTRODUCTION

Recent work has uncovered a set of bacterial regulatory RNAs, designated riboswitches, in which conserved cis–acting RNA elements located in the ‘leader region’ between the promoter and the regulated coding sequence respond to a variety of physiological signals without additional proteins or co-factors (1). Typical riboswitches are composed of two domains: the aptamer or ligand–sensing domain and the expression platform (2). The aptamer binds the cognate ligand and initiates a series of structural rearrangements that affect the fate of the downstream gene through structural modulation of the expression platform. Regulation of gene expression by these conserved leader RNAs can occur by transcription attenuation, modulation of translation initiation and effects on RNA stability (2,3).

A large number of genes that control essential processes are under riboswitch regulation in Bacillus subtilis (1). One of the earliest identified riboswitches, designated the L box, regulates transcription of the lysC gene in response to lysine (lys) (4–6). L box leader RNAs have been found upstream of a variety of genes involved in lys biosynthesis, transport and utilization (6–8). Lys biosynthesis in most bacteria and some plants occurs through the diaminopimelate pathway. The first step of lys biosynthesis is the phosphorylation of aspartate by aspartokinase. There are three differentially regulated aspartokinase isozymes in Escherichia coli and B. subtilis. However, only aspartokinase II, the product of the lysC gene, responds to lys (9). Formation of aspartate-4-phosphate is critical, as it is also a precursor for methionine and threonine biosynthesis (9). Other L box-regulated genes synthesize metabolites in the lys pathway that are required for other essential physiological processes, such as synthesis of cell wall peptidoglycan (10) and formation of bacterial endospores (11). Additional phylogenetic analyses suggest that L box leader RNAs are also involved in regulation of genes responsible for lys transport and the regulation of cellular pH (6,8).

When lys concentrations are high, lys binds to the B. subtilis lysC leader RNA and promotes the formation of an intrinsic terminator that prevents transcription of the lysC coding region (4,5). Low lys concentrations result in the formation of an alternate antiterminator structure that allows transcription to continue (Figure 1). In contrast, the E. coli lysC leader RNA is predicted to regulate expression at the translational level through the formation of a Shine–Dalgarno sequestering helix when lys concentrations are high (4,6,9). The lys analog aminoethylcysteine (AEC) is 10-fold less effective...
than lys in promotion of \textit{B. subtilis} \textit{lysC} termination \textit{in vitro} (4). AEC differs from lys by a single substitution of carbon with sulfur (Figure 3); the observed differential sensitivity demonstrates specific lys recognition by the \textit{lysC} leader RNA (4). Lys analog-binding studies using the aptamer region of the \textit{lysC} leader RNA further suggested that leader RNA binding is specific for lys (12).

Previous studies of riboswitch specificity identified several unique features adopted by riboswitch RNAs to recognize their cognate ligands. For example, the purine responsive riboswitches use canonical Watson–Crick base pairing for recognition (13). A single mutation of a conserved base in the guanine responsive riboswitch results in a specificity switch that allows recognition of adenine (14). Natural variants of the guanine and adenine riboswitches were identified that selectively bind 2′-deoxyguanosine (dG) and discriminate against guanine (15). The discovery of these variants demonstrates that riboswitch RNAs can use similar RNA architectures to recognize different molecules.

Several riboswitches use similar mechanisms to recognize nucleotide-like molecules. In each example, the RNA uses common features such as the face of the nucleotide to assist in recognition. Recognition of S-adenosylmethionine

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Sequence and predicted secondary structural model of the \textit{B. subtilis} \textit{lysC} leader RNA. Boxed letters and numbers indicate the terminator (T), antiterminator (AT), anti-antiterminator (AAT) and helices 1–6. Residues shown in red are found in 100% of the sequences analyzed and blue residues are found in >80% (4). Green residues connected by dashed lines indicate sequences that show extensive covariation (4) and form a tertiary interaction in the crystal structure (23). Arrows identify the \textit{lysC} variants constructed for this study. Bold nucleotides indicate residues shared between the terminator and antiterminator elements. Purple nucleotides are complementary to the DNA oligonucleotide probe used in RNase H cleavage assays.}
\end{figure}
Another common mechanism for ligand recognition incorporates divalent metal ions for both binding and recognition of negatively charged groups. RNAs that have been shown to use this method include the S box (SAM-I) riboswitch (16). Similarly, the aminopyrimidine ring of the thiamine pyrophosphate (TPP) molecule is recognized by the THI-box RNA through molecular stacking with conserved residues (17). The nucleotide face of the cyclic di-GMP (c-di-GMP) molecule is also recognized using asymmetric RNA interactions (18).

Although the RNAs recognize similar features of different ligands, each riboswitch utilizes a different mechanism for recognition.

A glycine riboswitch (20). Currently, the role of K+ in the binding and function of L box leader RNAs has not been established, but it was suggested that it might assist in lys recognition (23).

With the variety of genes under L box control, it is essential that the RNA selectively binds lys and efficiently regulates expression under appropriate conditions. The current structural model of the lys-binding site does not clearly demonstrate how the RNA can specifically recognize lys and discriminate against substitutions in the ligand. Previous studies of lys analog recognition focused on a subset of available compounds (12). In this study, we test a broad range of lys analogs and identify mutations that affect ligand recognition; these mutations reveal molecular interactions responsible for recognition and specificity. We also investigate the role of K+ in the binding site and the potential effects on leader RNA function.

**MATERIALS AND METHODS**

**Construction of DNA templates**

Templates for in vitro transcription by *B. subtilis* RNA polymerase (RNAP) were generated by fusion of the *B. subtilis glyQS* promoter sequence to the lysC leader RNA sequence. PCR was carried out using DNA oligonucleotide primers that included the glyQS promoter and hybridized within the lysC leader region. A transcriptional fusion plasmid, pFG328, with a wild-type lysC insertion (4), was the DNA source for PCR amplification using *Taq* DNA polymerase as per the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The resulting PCR fragment contained the lysC leader template downstream of the glyQS promoter such that transcription would start at +17 relative to the lysC transcription start-site and included lysC sequence extending to 14 nt downstream of the leader region termination site. The transcription start site was designed to allow transcription initiation with the dinucleotide ApC and a halt at position G42 (relative to the native lysC transcript) during transcription in the absence of cytidine 5’-triphosphate (CTP). The 3’-end of the construct included an additional 106 bp of random sequence to allow resolution between terminated (252 nt) and readthrough (372 nt) transcripts. PCR products were purified using a QIAquick PCR clean up kit (Qiagen, Chatsworth, CA, USA) and sequenced by Genewiz (South Plainfield, NJ, USA).

DNA templates used for T7 RNAP transcription were generated using complementary pairs of overlapping DNA oligonucleotides as previously described (25,26).
Briefly, the 5' pair contained the phage T7 RNAP promoter sequence fused to the +18 position of the \( \text{lysC} \) leader region. The remaining complementary pairs contained the wild-type \( \text{lysC} \) sequence, each with a 5-nt 3' overhang complementary to the 5' region of the adjacent pair. The terminal pair contained the 3' leader RNA sequence that ended at +234, 3 nt prior to the 5' position of the transcription terminator. This position was selected so that helix 1 formation could be monitored without the competing antiterminator structure. Each internal oligonucleotide pair was phosphorylated using T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA). The pairs were then mixed, incubated at 95°C and slow cooled to room temperature for annealing. The paired oligonucleotides were ligated using T4 DNA ligase per the manufacturer's instructions (New England Biolabs, Beverly, MA, USA). The resulting DNA template was amplified using the flanking 5' and 3'DNA oligonucleotides as primers for PCR using Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA). The final template DNA was purified and sequenced as described above.

**Site-directed mutagenesis**

The transcriptional fusion vector pFG328 containing the wild-type \( \text{lysC} \) DNA template (4) was used as a template for oligonucleotide-directed mutagenesis. DNA oligonucleotides containing the desired mutations were designed as primers for PCR amplification of pFG328-\( \text{lysC} \) DNA using Pfu DNA polymerase (Stratagene, La Jolla, CA, USA). The resulting products were subjected to digestion with DpnI (New England Biolabs, Beverly, MA, USA) to remove the starting wild-type template and introduced into XL-2 blue ultracompetent cells by transformation as per the manufacturer's instructions (Stratagene, La Jolla, CA, USA). The plasmid DNA was isolated using a Promega Wizard prep kit and sequenced to confirm the mutations (Genewiz, South...
**In vitro transcription termination assays**

Single round transcription of the wild-type *lysC* leader RNA template was performed as previously described by Grundy *et al.* (27). A reaction mixture of 20 mM Tris–HCl, pH 8, 20 mM NaCl, 10 mM MgCl₂, 100 μM EDTA, 150 μM ApC (Sigma), 2.5 μM GTP and ATP, 0.75 μM UTP, [γ-32P]UTP (GE Healthcare; 0.25 μM, 800 Ci/mmol [30 TBq/mmol]), DNA template (10 nM) and His-tagged purified *B. subtilis* RNAP (6 nM) (28) was incubated for 15 min at 37°C. CTP was excluded from the reaction mixture to generate a transcriptional halt at +42 (relative to the native *lysC* transcription start site). After incubation, Heparin was added to block reinitiation and synchronize transcript synthesis. KCl (27 mM) was added where indicated. Lys or lys analogs were added, and elongation was resumed by the addition of 10 μM rNTPs and 40 mM MgCl₂ followed by incubation for 15 min at 37°C. The transcription reactions were stopped by phenol/chloroform extraction, and the products were resolved by denaturing PAGE and visualized by PhosphorImager analysis (Molecular Dynamics). Percent termination represents terminated transcripts relative to the total amount of RNA transcribed; this value was plotted as a function of ligand concentration. Non-linear regression analysis was used to determine the concentration required for half-maximal termination (Trm½) (SigmaPlot, Systat Software). Reactions were performed in duplicate and reproducibility was ±5%.

**RESULTS**

**Wild-type *lysC* transcription termination in response to lys analogs**

*In vitro* transcription of the wild-type *lysC* leader by *B. subtilis* RNAP in the presence of lys or lys analogs (Table 1) was used to identify features of the lys molecule required for ligand-dependent transcription termination. Lys and lys analogs with the ability to promote termination of the *lysC* leader RNA were further tested in *in vitro* using a broad range of ligand concentrations (0–12.8 mM) (Table 1). Transcription of the *lysC* leader region in the presence of lys (10 mM) resulted in 96% termination and the concentration required for Trm½ was 0.17 mM. In *in vitro* transcription in the presence of 11 different lys analogs (added at 10 mM final concentration) showed that alterations to the main chain functional groups, as seen in ω-lys and diaminopentane, resulted in a reduction of ligand-dependent termination (Table 1). The Trm½ for both ω-lys and diaminopentane was >13 mM. These results suggest that changes to functional groups that participate in RNA–ligand interactions are

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**Table 1. *In vitro* transcription termination of wild-type *lysC* leader in the presence of lys or lys analogs**

| Ligand Wild-type | Term. | Ratio | Trm½ (mM) |
|------------------|-------|-------|----------|
| **Lysine** |       |       |          |
| None            | 31    |       | 0.17 ± 0.02 |
| Lys             | 96    | 3.2   | >13      |
| n-lys           | 46    | 1.5   | >13      |
| Diaminopentane | 43    | 1.4   | >13      |
| **Alkyl chain** |      |       |          |
| AEC             | 84    | 2.7   | 0.80 ± 0.11 |
| Hydroxy lys     | 35    | 1.1   | >13      |
| t-ornithine     | 32    | 1.0   | ND       |
| **E-amino group** |    |       |          |
| Lys methyl      | 73    | 2.4   | 1.3 ± 0.40 |
| t-arg           | 35    | 1.1   | ND       |
| **Carboxyl group** |   |       |          |
| lysNH           | 90    | 2.9   | 0.30 ± 0.02 |
| lysOH           | 87    | 2.8   | 1.9 ± 0.34 |
| lysEE           | 76    | 2.5   | 1.0 ± 0.25 |

*Term. (term.) is the percent of RNAs terminated in the presence of 10 mM ligand relative to the total amount of RNA transcribed. Ratio is the ratio of percent terminated transcripts in the presence of 10 mM ligand to the percent terminated transcripts in the absence of ligand. *Trm½ is the concentration of ligand required to promote 50% termination. ND, not determined.*
The increase in Trm1/2 in the presence of lys methyl causes reduced ligand recognition. The Trm 1/2 in the presence of AEC (84%) suggests that the methyl addition to the side chain of the molecule is deleterious to ligand recognition. The Trm1/2 in the presence of lysNH as compared to lys methyl and AEC; this may be indicative of a larger interaction between the leader RNA and the carboxyl group in the binding pocket (Figure 2). Substitution of G111 with C resulted in reduced termination (12%) in the absence of ligand as compared to wild-type (Table 1; 31%) and caused a reduction in sensitivity to lys and AEC (Table 2). Each G111 variant exhibited a reduction in termination in the presence of lys and AEC. However, the G111A substitution resulted in a 2-fold increase in termination in response to DAP (Table 2), which is inactive in the context of the wild-type lysC sequence (Table 1). Similarly, the G111U substitution resulted in a 5-fold increase in termination in response to lys methyl, as compared to the no ligand control (Table 2). The reduction in lys-dependent termination suggests that G111 plays a pivotal role in lys recognition and/or binding. However, the termination response in the presence of DAP (G111A) and lys methyl (G111U) suggests that G111 is not required for termination. The sensitivity of G111A and G111U to ε-amino-modified analogs is in good agreement with the published crystal structure, as G111 is positioned nearest the ε-amino group of the lys molecule (23).

Unlike G111, in which no substitution allowed normal function, A112 was amenable to substitution with a guanine residue, exhibiting a ligand response similar to that of wild-type, although termination in the presence of ε-amino and carboxyl modified analogs was reduced (Tables 1 and 2). When A112 was substituted with either pyrimidine base (Table 2), there was a decrease in termination in both the presence and absence of ligand (~7% for A112C and ~19% for A112U). Both A112 and G111 were intolerant to pyrimidine substitutions, which suggests that purines are required for ligand recognition and lys-dependent termination. In contrast to A112G, the purine–purine substitution of G111 with A was deleterious to the function of the RNA. This suggests that there are specific interactions between G111 and the lys molecule, which directly contribute to lys-dependent termination.

**Effects of substitutions at G111 and A112**

In an effort to identify sequence elements within the lysC leader that are important for ligand recognition, we used site-directed mutagenesis to probe RNA requirements for lys-dependent termination. The nucleotides chosen for mutation are highly conserved (Figure 1) and predicted to interact with lys (Figure 2). Universally conserved G111 and A112 are positioned nearest to the ε-amino group in the binding pocket (Figure 2). Modification of the ε-amino group was tolerated only in the context of the addition of a methyl group, as in lysine methyl (lys methyl). Transcription in the presence of lys methyl resulted in a Trm1/2 of 1.3 mM, an 8-fold increase as compared to lys. The increase in Trm1/2 in the presence of lys methyl suggests that the methyl addition to the ε-amino group is deleterious to ligand recognition. The Trm1/2 in the presence of lysOH as compared to lysEE emphasizes the importance of functional group recognition as compared to minor alterations in ligand length. This indicates that both the identity of the molecular substitution and additional interactions within the binding pocket affect ligand recognition.
region of the lys molecule. Crystal structure analysis revealed base pairing between G40 and C110 of the leader RNA, which is in agreement with previous studies that identified mutations at this position that result in deregulation of the lysC leader and cause AEC resistance (29). The conserved G40–C110 and G144–U170 base pairs were substituted with other canonical base pairs and tested for termination in the presence of lys or lys analogs. The G40U–C110A substitution inhibited lys-dependent termination (Table 3). Lys or lys analogs with substitutions in the side chain (AEC) or ε-amino group were not recognized by the G40A–C110U variant (Table 3). However, termination of the G40A–C110U variant was observed in the presence of carboxyl-modified analogs. Transcription of the G40A–C110U variant in the presence of lysNH, which replaces a carboxyl oxygen with an amide, resulted in a reduction in termination (Table 3) as compared to wild-type (Table 1). Termination of the G40A–C110U variant increased 4-fold in the presence of lysOH (75%) as compared to no ligand control (Table 4). The Trm1/2 exhibited by the G40A–C110U variant in the presence of lysNH was 2.1 mM as compared to >13 mM for lys (Table 4). The Trm1/2 exhibited by the G40A–C110U variant in the presence of lysOH was similar to wild-type; however, the Trm1/2 was increased, as compared to wild-type, in the presence of lys (Table 4). The response to lysOH and lack of response to lys suggests that the G40A–C110U mutations cause a change in ligand recognition specificity. Termination of the G40A–C110U variant in the presence of lysNH, as compared to lysOH, indicates that the hydroxyl substitution at the carbonyl more effectively promotes termination than an amide at the same position. It is plausible that the change in specificity is due to the formation of new molecular interactions between G40A and the amide or hydroxyl group of the lys analog.

| Lysine modification | Ligand | Variant |
|---------------------|--------|---------|
| G111C               | Term.  | Ratio  |
| None                | 12     | 14     |
| Lys                 | 13     | 1.1    |
| α-lys               | 16     | 1.3    |
| Diaminopentane      | 15     | 1.2    |
| Global              |        |        |
| AEC                 | 12     | 1.0    |
| Hydroxy lys         | 13     | 1.1    |
| t-ornithine         | 13     | 1.1    |
| Alkyl chain         |        |        |
| Lys methyl          | 13     | 1.1    |
| t-arg               | 14     | 1.2    |
| DAP                 | 14     | 1.2    |
| ε-amino group       |        |        |
| LysOH               | 13     | 1.1    |
| lysEE               | 16     | 1.3    |
| Carboxyl group      |        |        |
| lysNH               | 13     | 1.1    |
| lysOH               | 13     | 1.1    |
| lysEE               | 16     | 1.3    |

In vitro transcription of G40–C110 lysC leader variants in the presence of 10 mM lys or lys analogs

| Lysine modification | Ligand | Variant |
|---------------------|--------|---------|
| U–A                 | Term.  | Ratio  |
| None                | 26     | 17     |
| Lys                 | 24     | 0.92   |
| α-lys               | 34     | 1.3    |
| Diaminopentane      | 25     | 0.96   |
| Global              |        |        |
| AEC                 | 22     | 0.85   |
| Hydroxy lys         | 24     | 0.92   |
| ε-amino group       |        |        |
| Lys methyl          | 29     | 1.1    |
| t-ornithine         | 34     | 1.3    |
| Carboxyl group      |        |        |
| lysNH               | 26     | 1.0    |
| lysOH               | 33     | 1.3    |
| lysEE               | 24     | 0.92   |

Table 2. In vitro transcription of G111 and A112 lysC leader variants in the presence of lys or lys analogs

Table 3. In vitro transcription of G40–C110 lysC leader variants in the presence of lys or lys analogs

Termination (term.) is the percent of RNAs terminated relative to the total transcription.
Ratio is the ratio of percent terminated transcripts in the presence of 10 mM ligand to the percent terminated transcripts in the absence of ligand.
that base pairing is required at this position (Table 5). Variants that maintained base pairing between positions 144 and 170 showed an increase in termination in the presence of lys methyl, lysNH and lysEE. With the exception of the A–U and C–G constructs, all other variants resulted in termination efficiencies lower than that of wild-type in the presence of lys methyl, lysNH and lysEE (Tables 1 and 5). The C–G variant (G144C–U170G) showed a 14% increase in termination in the absence of ligand (Table 5) as compared to wild-type (Table 1), which suggests that the nucleotide identity at position 144 affects the unliganded RNA structure (C–G). In contrast to the C–G variant, the G–C variant (G144–U170C) resulted in a reduction of ligand-dependent termination as compared to wild-type in the absence of ligand (G–C) (Tables 1 and 5). Termination of the G–C variant in the presence of lys and lys analogs was reduced as compared to wild-type. These results indicate that the U170C mutation does not abolish function of the RNA but perturbs ligand recognition. Transcription of the G144A–U170 variant in the presence of lysEE resulted in increased termination (94%) (Table 5; A–U) as compared to wild-type termination in the presence of lysEE (76%) (Table 1). The Trm1/2 of the G144A variant for lysEE was 0.85 mM, similar to that of wild-type (Table 4). However, Trm1/2 of the G144A variant in the presence of lys was >13 mM, suggesting a loss in lys recognition (Table 4). The G144A substitution, a G–U to A–U base pair exchange, caused an alternate ligand specificity where the variant leader RNA responds to lysEE and discriminates against lys. Substitution of the G144A–U170 pair with U–A resulted in a reduction of termination efficiency in the presence of carboxyl-modified analogs; however, ligand specificity was similar to that of the A–U variant. Recognition of lys methyl by the G144–U170 variants is surprising as these positions were not predicted to reside near the ε-amino group. One explanation is that the G144–U170 base pair is not primarily responsible for ε-amino group recognition. Termination in the presence of lys analogs as opposed to lys and AEC suggests that substitution of the G40–C110 and G144–U170 base pairs resulted in a loss of lys recognition. However, G144–U170 variants retained specificity for ε-amino and carboxyl-modified analogs while G40A–C110U exhibited specificity for analogs with carboxyl modifications. Together, these results indicate that specific mutations of the RNA sequence can result in alternate ligand specificity whereby the leader RNA responds to lys analogs while discriminating against lys.

Table 4. Termination efficiency of lysC leader variants in the presence of lys or lys analogs

| Ligand    | Variant       | A112G | G40A–C110U | G144A–U170 |
|-----------|---------------|-------|------------|------------|
| lys       | 0.38 ± 0.07   | >13   | >13        |
| AEC       | 1.2 ± 0.20    | ND    | ND         |
| lysOH     | ND            | 2.1 ± 0.20 | ND         |
| lysEE     | ND            | 0.85 ± 0.10 |

*Trm1/2 is the concentration of ligand required to promote 50% termination.

Table 5. In vitro transcription termination of G144–U170 lysC leader variants in the presence of 10 mM lys or lys analogs

| Lysine modification | Ligand       | U–G Term. | Ratio | G–G Term. | Ratio | C–G Term. | Ratio | G–C Term. | Ratio | A–U Term. | Ratio | U–A Term. | Ratio |
|---------------------|--------------|-----------|-------|-----------|-------|-----------|-------|-----------|-------|-----------|-------|-----------|-------|
| Global              | lys          | 33        | 1.0   | 22        | 1.0   | 45        | 1.0   | 26        | 1.1   | 22        | 1.0   | 19        | 0.9   |
|                     | ε-lys        | 41        | 1.2   | 28        | 1.3   | 48        | 1.1   | 29        | 1.1   | 26        | 1.2   | 23        | 1.1   |
|                     | Diaminopentane | 41       | 1.3   | 23        | 1.0   | 69        | 1.5   | 35        | 1.3   | 38        | 1.7   | 25        | 1.2   |
| Alkyl chain         | AEC          | 30        | 0.9   | 19        | 0.9   | 41        | 0.9   | 24        | 0.9   | 23        | 1.0   | 18        | 0.9   |
|                     | Hydroxy lys  | 38        | 1.2   | 23        | 1.0   | 51        | 1.1   | 24        | 0.9   | 30        | 1.4   | 18        | 0.9   |
|                     | 1-ornithine  | 28        | 0.8   | 21        | 1.0   | 40        | 0.9   | 24        | 0.9   | 22        | 1.0   | 17        | 0.8   |
| ε-amino group       | Lys methyl   | 50        | 1.5   | 24        | 1.1   | 92        | 2.0   | 70        | 2.7   | 68        | 3.1   | 66        | 3.1   |
|                     | 1-arg        | 30        | 0.9   | 23        | 1.0   | 40        | 0.9   | 21        | 0.8   | 22        | 1.0   | 18        | 0.9   |
|                     | DAP          | 32        | 1.0   | 21        | 1.0   | 43        | 1.0   | 25        | 1.0   | 23        | 1.0   | 16        | 0.8   |
| Carboxyl group      | lysNH        | 49        | 1.5   | 24        | 1.1   | 87        | 1.9   | 57        | 2.2   | 73        | 3.3   | 63        | 3.0   |
|                     | lysOH        | 47        | 1.4   | 20        | 0.9   | 67        | 1.5   | 34        | 1.3   | 41        | 1.9   | 36        | 1.7   |
|                     | lysEE        | 72        | 2.1   | 24        | 1.1   | 91        | 2.0   | 49        | 1.9   | 94        | 4.2   | 72        | 3.4   |

*Termination (term.) is the percent of RNAs terminated relative to the total transcription.

*Ratio is the ratio of percent terminated transcripts in the presence of 10 mM ligand to the percent terminated transcripts in the absence of ligand.
K+ decreases the concentration of lys required for transcription termination

The B. subtilis lysC leader RNA does not require K+ for lys-dependent termination in vitro (4,5). However, recent analyses suggest that K+ is important for lys recognition and affinity (7,30). We, therefore, tested the effect of addition of K+ to our purified in vitro transcription system. In the presence of K+, the wild-type leader RNA showed a 5-fold decrease in Trm1/2 for lys (Fig. 4A). Other monovalent (NaCl) and divalent salts (CaCl2) were also assayed to test whether the observed effect was specific to K+ and no observable change in Trm1/2 was detected (our unpublished results). The 5-fold decrease in Trm1/2 for lys in the presence of K+ suggests that K+ plays a key role in lys-dependent termination by increasing the affinity of the RNA for lys.

Variant leader RNAs and lys analogs were further tested to determine if the K+ is involved in ligand recognition. The A112G variant, which does not show altered ligand recognition but exhibited a 2-fold decrease in lys-dependent termination relative to the wild-type (Table 2), exhibited no observable change in lys sensitivity in response to K+ (Figure 4A). The lack of response to the presence of K+ may be the result of changes in the structure of the binding pocket imposed by the A112G mutation. Based on the position of K+ in the structure, it is plausible that the G40–C110 or G144–U170 base pairs of the B. subtilis lysC binding pocket interact directly with K+ (Figure 2). The G40A–C110U variant, which exhibited increased termination only in the presence of lysOH (Table 3), exhibited no change in Trm1/2 for lysOH in the presence of K+ (Figure 4B). Similarly, the G144A variant, which responds to lysOH, showed no reduction in Trm1/2 for lysEE in the presence of K+ (Figure 4B). Transcription of the G144A variant in the presence of lys demonstrated that K+ was unable to enhance lys recognition (our unpublished results). These results indicate that K+ does not play a role in the specificity of ligand recognition but rather affects affinity of the wild-type leader RNA for lys.

lysC leader RNA variants undergo ligand-dependent structural changes

Structural rearrangement of the leader RNA when the regulatory ligand is present is the hallmark of riboswitch regulation. This structural change is crucial as it directly controls gene expression. The current model of the lysC leader RNA structural transition indicates that formation of the transcription terminator requires the stabilization of the anti-antiterminator (AAT; Figure 1) through the formation of helix 1 (4). To monitor the structural change of the variant leader RNAs in response to lys analogs, we used RNase H probing of helix 1. Addition of a DNA oligonucleotide complementary to the 3' side of helix 1 will result in cleavage only when helix 1 is unpaired; stabilization of the helix prevents binding of the oligonucleotide, which results in protection of the RNA from cleavage by RNase H, an endonuclease that is specific for RNA–DNA hybrids. In the absence of ligand, 31% of the wild-type RNAs were protected from RNase H cleavage as compared to 89% protection in the presence of lys (Figure 5, lanes 1 and 2). These results indicate that the presence of lys promotes the formation of helix 1. When wild-type RNAs were transcribed in the presence of high concentrations of AEC, 84% protection was observed (Figure 5, lane 3). Similarly, wild-type RNAs exhibited 82% protection in the presence of lysOH and 60% protection in the presence of lysEE (Figure 5, lanes 4 and 5). The reduced protection of wild-type helix 1 in the presence of lys analogs, as compared to lys, is consistent with lower wild-type termination in vitro in the presence of lys analogs. For the A112G variant, 48% of the transcribed RNAs were protected from cleavage in the no ligand control as compared to 65% in the presence of lys (Figure 5, lanes 6 and 7). A reduction in protection of the A112G variant, as compared to wild-type, was observed in the presence of AEC, lysOH, and lysEE (Figure 5, lanes 8–10). This demonstrates that protection

![Figure 4](https://academic.oup.com/nar/article-abstract/40/12/5706/2414815)

**Figure 4.** In vitro transcription of wild-type and variant lysC leader RNAs in the presence and absence of potassium. Wild-type or variant leader RNAs were transcribed in the presence or absence of 2mM KCl. Open symbols represent transcription in the absence of KCl, filled symbols represent transcription in the presence KCl. (A) Wild-type (circles) and A112G (squares) templates transcribed in the presence of lys. (B) Transcription of the G40A-C110U variant in the presence of lysOH (circles) and the G144A-U170 variant transcribed in the presence of lysEE (squares).
Figure 5. RNase H cleavage of wild-type and variant lysC leader RNAs. Radiolabeled RNAs were transcribed in the presence of 7.5 mM lys, AEC, lysOH and lysEE and incubated with a DNA oligonucleotide complementary to the 3' side of helix 1 to allow hybridization followed by RNase H digestion. Helix 1 protection of wild-type in the presence of lys and lys analogs is shown in lanes 1–5; lanes 6–10, A112G variant; lanes 11–15, G40A–C110U variant; and lanes 16–20, G144A variant. FL, full-length transcripts protected from RNase H cleavage. C, transcripts cleaved by RNase H. Percent protection is amount of full-length RNAs relative to total amount of RNA transcribed.

DISCUSSION

Previous studies of riboswitch specificity have identified RNA elements involved in ligand recognition (25,30,31). However, these features are variable and often specific to the RNA and its cognate ligand. Structural analysis of ligand-bound riboswitches has revealed mechanistic themes employed for ligand recognition. Ligand features such as negatively charged groups and nucleotide-like structures are targets for recognition. Shape complementarity, base pairing, nucleotide stacking and metal ion coordination are known mechanisms of ligand recognition by riboswitches. The molecular structure of lys makes a number of these mechanisms impossible.

Recent analysis of the aptamer region of the T. maritima L box leader RNA has identified the nucleotide composition of the lys-binding pocket. Although the nucleotides that directly contact lys were identified, the molecular interactions and their effect on function are poorly understood. In this study, we use the lys-responsive B. subtilis lysC leader RNA to analyse recognition determinants and their effects on ligand-dependent structural changes and transcription termination.

Analysis of transcription in vitro in the presence of lys analogs correlates with the crystal structure, which suggested that lys recognition relies heavily on shape complementarity between the ligand and the RNA-binding pocket (23). This feature seems to be a common characteristic of riboswitch RNAs and is also observed in the c-di-GMP and TPP riboswitches (17,18). For the lysC leader RNA, the effective concentration for each analog is dependent on its structural similarity to lys. The primary determinant for ligand recognition by the lysC leader RNA appears to be the ability of the ligand to fit into the lys-binding pocket. This suggests that shape recognition is likely to be the first step in discrimination between lys and similar molecules. Analogs that maintain the overall shape of the molecule, with minor molecular substitutions such as those found in AEC and lysNH, were more effective at promoting both the ligand-dependent structural changes and transcription termination. Changes to the chemistry and charge of the molecule at either the carboxyl or e-amino termini were tolerated to some degree, and groups at these positions could be replaced with other functional groups that make similar interactions. It is possible that molecular extensions such as a methyl group (lys methyl) or ethyl ester group (lysEE) can fit into small channels in the RNA pocket and promote termination at concentrations similar to those required for lys. This suggests that the lys-binding pocket has evolved to bind the lys molecule with high affinity and specificity. These results demonstrate that the B. subtilis lysC leader RNA is optimized for lys-dependent regulation.

Mutational analysis uncovered features of the RNA that are essential for lys recognition. G111 is critical for lys-dependent regulation. Previous studies have indicated that this residue is involved in structurally significant base
triple interactions that may be essential for formation of the lys-binding pocket (23). The data presented here provide evidence that G111 is required for formation of the lys-binding pocket as well as lys recognition. It is possible that G111 must be precisely positioned to interact with other nucleotides within the RNA and the bound lys molecule. Additionally, our data suggest that A112 is not essential for ligand recognition but is primarily involved in lys affinity. This effect on affinity may be achieved through extensive contacts with A112 that stabilize the structure of the lys-binding pocket. Variants of the lys-binding pocket near the carboxyl region of the lys-molecule (G40A–C110U and G144A–U170) exhibited preferential specificity for certain lys analogs and discriminated against lys. The alternate specificity of leader RNA variants suggests that each base pair is responsible for the recognition of molecular features of the lys carboxyl group. Additionally, mutations in the carboxyl region of the binding pocket did not inhibit recognition of the ε-amino region, as G144–U170 variants retained the ability to recognize lys methyl. The leader RNA is able to use alternate molecular interactions with lys analogs to promote termination. However, the increased concentration requirement for lys analogs suggests that the new RNA–analog interactions do not perfectly mimic the interactions with lys. This analysis confirms that the sequence of lysC leader RNA is optimized for lys recognition as all of the variants in this study, with the exception of A112G, exhibited a loss of lys-dependent termination. In total, these data indicate that recognition of lys by the L box riboswitch is a modular process in which individual nucleotides recognize independent features of the lys molecule.

The presence of a K+ ion in the L box crystal structure (23) raised questions about the role of this ligand in binding. Analysis of the effects of the addition of K+ to the in vitro transcription termination assay revealed that the potassium ion may increase the affinity of the leader RNA for lys. The increased affinity was observed only in the presence of the wild-type RNA and lys. This suggests that both the sequence and structure of the binding pocket are necessary for stimulation of tighter lys binding by K+. Additional studies are required to determine the direct effects of K+ on lys binding.

The discovery of new riboswitches and characterization of RNA–ligand complexes continue to diversify our understanding of the mechanisms used by RNAs to recognize and bind small molecules. Small molecule recognition by riboswitches is essential to regulation, as gene expression must occur only in response to the appropriate stimulus. Recognition of the appropriate ligand by riboswitch RNAs can occur in a variety of ways with some common themes distributed amongst the different classes. The lysC leader RNA not only utilizes some common mechanisms, such as shape complementarity and metal ion assistance for lys binding and recognition, but also makes nucleotide-specific interactions for ligand identification. These interactions differ from the mechanism used by the glycine riboswitch to recognize similar amino acid features (19). The alternate specificities exhibited by lysC variants demonstrate that the leader RNA uses a unique arrangement of contacts to recognize the ligand and regulate gene expression. This characterization has provided a more detailed view of how the lysC leader RNA specifically recognizes the lys molecule and contributes to our current understanding of small molecule recognition by RNAs. However, the specific features responsible for lys binding affinity are still unclear and require further analysis.

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REFERENCES

1. Henkin, T.M. (2008) Riboswitch RNAs: using RNA to sense cellular metabolism. Genes. Dev., 22, 3383–3390.
2. Smith, A.M., Fuchs, R.T., Grundy, F.J. and Henkin, T.M. (2010) Riboswitch RNAs: regulation of gene expression by direct monitoring of a physiological signal. RNA Biol., 7, 104–110.
3. Cheeh, M.T., Watcher, A., Sudarsan, N. and Breaker, R.R. (2007) Control of alternate RNA splicing and gene expression by eukaryotic riboswitches. Nature, 447, 497–501.
4. Grundy, F.G., Lehman, S.C. and Henkin, T.M. (2003) The L box regulon: lysine sensing by leader RNAs of bacterial lysine biosynthesis genes. Proc. Natl Acad. Sci., 100, 12057–12062.
5. Sudarsan, N., Wickiser, J.K., Nakamura, S., Ebert, M.S. and Breaker, R.R. (2003) An mRNA structure in bacteria that controls gene expression by binding lysine. Genes. Dev., 17, 2688–2697.
6. Rodionov, D.A., Vitreschak, A.G., Mironov, A.A. and Gelfand, M.S. (2003) Regulation of lysine biosynthesis and transport genes in bacteria: yet another RNA riboswitch? Nucleic Acids Res., 31, 6748–6757.
7. Serganova, A. and Patel, D.J. (2009) Amino acid recognition and gene regulation by riboswitches. Biochim. Biophys. Acta., 1792, 592–611.
8. Jorth, P. and Whiteley, M. (2010) Characterization of a novel riboswitch-regulated lysine transporter in Aggregatibacter actinomycetemcomitans. J. Bacteriol., 192, 6240–6250.
9. Parke, J.C., Akrım, M. and Mejean, V. (1998) The leader sequence of the Escherichia coli lysC gene is involved in the regulation of LysC synthesis. FEMS Microbiol. Lett., 169, 165–170.
10. Pavlik, M.S. and Jacobs, W.R. (1996) Biosynthesis of diaminopimelate, the precursor of lysine and a component of the peptidoglycan, is an essential function of Mycobacterium smegmatis. J. Bacteriol., 178, 6496–6507.
11. Forman, M. and Aronson, A. (1972) Regulation of dipicolinic acid biosynthesis in sporulating Bacillus cereus characterization of enzymatic changes and analysis of mutants. Biochem. J., 126, 503–513.
12. Blount, K.F., Wang, J.X., Lim, J., Sudarsan, N. and Breaker, R.R. (2007) Antibacterial lysine analogs that target lysine riboswitches. Nat. Chem. Biol., 3, 44–49.
13. Noeske, J., Richter, C., Grundl, M.A., Nasirli, H.R., Schwalbe, H. and Wöhner, J. (2005) An intramolecular base triple as the basis of ligand specificity and affinity in the guanine- and adenine-sensing riboswitch RNAs. Proc. Natl Acad. Sci., 102, 1372–1377.
14. Mandal, M.M. and Breaker, R.R. (2004) Adenine riboswitches and gene activation by disruption of a transcription terminator. Nat. Struct. Mol. Biol., 11, 29–35.
15. Kim, J., Roth, A. and Breaker, R.R. (2007) Guanine riboswitch variants from Mesoplasma florum selectively recognize 2’-deoxyguanosine. Proc. Natl Acad. Sci., 104, 16092–16097.
16. Lu, C., Ding, F., Chowdhury, A., Pradhan, V., Tomsic, J., Holmes, W.M., Henkin, T.M. and Ke, A. (2010) SAM recognition and conformational switching mechanism in the Bacillus subtilis yit J S box/SAM-I riboswitch. J. Mol. Biol., 404, 803–818.
17. Edwards, T.E. and Ferré-D’Amare, A.R. (2006) Crystal structures of the Thi-Box riboswitch bound to thiamine pyrophosphate analogs reveal adaptive RNA-small molecule recognition. Structure, 14, 1459–1468.
18. Smith, K.D., Lipchock, S.V., Livingston, A.L., Shanahan, C.A. and Strobel, S.A. (2010) Structural and biochemical determinants of ligand binding by the c-di-GMP Riboswitch. Biochemistry, 49, 7351–7359.
19. Huang, L., Serganov, A. and Patel, D.J. (2010) Structural insights into ligand recognition by a sensing domain of the cooperative glycine riboswitch. Mol. Cell., 40, 774–786.
20. Edwards, T.E., Klein, D.J. and Ferré-D’Amare, A.R. (2007) Riboswitches: small-molecule recognition by gene regulatory RNAs. Curr. Opin. Struct. Biol., 17, 273–279.
21. Mandal, M., Lee, M., Barrick, J.E., Weinberg, Z., Emilsson, G.M., Ruzzo, W.L. and Breaker, R.R. (2004) A glycine-dependent riboswitch that uses cooperative binding to control gene expression. Science, 306, 275–279.
22. Garst, A.D., Héroux, A., Rambo, R.P. and Batey, R.T. (2008) Crystal structure of the lysine riboswitch regulatory mRNA element. J. Biol. Chem., 283, 22347–22351.
23. Serganov, A., Huang, L. and Patel, D.J. (2008) Structural insights into amino acid binding and gene control by a lysine riboswitch. Nature, 455, 1263–1268.
24. Blouin, S., Chinnappan, R. and Lafontaine, D.A. (2011) Folding of the lysine riboswitch: importance of peripheral elements for transcriptional regulation. Nucleic Acids Res., 39, 3373–3387.
25. McDaniel, B.A., Grundy, F.J. and Henkin, T.M. (2005) A tertiary structural element in S box leader RNAs is required for S-adenosylmethionine-directed transcription termination. Mol. Microbiol., 57, 1008–1021.
26. Yousef, M.R., Grundy, F.J. and Henkin, T.M. (2005) Structural transitions induced by the interaction between tRNA and the Bacillus subtilis glyQS T box leader RNA. J. Mol. Biol., 349, 273–287.
27. Grundy, F.J., Winkler, W.C. and Henkin, T.M. (2002) tRNA-mediated transcription antitermination in vitro: codon-anticodon pairing independent of the ribosome. Proc. Natl Acad. Sci., 99, 11121–11126.
28. Qi, Y. and Hulett, F.M. (1998) Pho−P and RNA polymerase σ70 holoenzyme are sufficient for transcription of Pho regulon promoters in Bacillus subtilis: Pho−P activator sites within the coding region stimulate transcription in vitro. Mol. Microbiol., 28, 1187–1197.
29. Lu, Y., Chen, N. and Paulus, H. (1991) Identification of accA mutations in Bacillus subtilis as nucleotide substitutions in the untranslated leader region of the aspartokinase II operon. J. Gen. Microbiol., 137, 1135–1143.
30. Serganov, A., Polonskaia, A., Phan, A.T., Breaker, R.R. and Patel, D.J. (2006) Structural basis for gene regulation by a thiamine pyrophosphate-sensing riboswitch. Nature, 441, 1167–1171.
31. Kim, J. and Breaker, R.R. (2008) Purine sensing by riboswitches. Biol. Cell, 100, 1–11.