Leader Peptide-mediated Transcriptional Attenuation of Lysine Biosynthetic Gene Cluster in *Thermus thermophilus*

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Taishi Tsubouchi, Reiko Mineki, Hikari Taka, Naoko Kaga, Kimie Murayama, Chiharu Nishiya, Hisakazu Yamane, Tomohisa Kuzuyama, and Makoto Nishiyama

From the §Biotechnology Research Center, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan and §BioMedical Research Center and ¶Atopy Research Center, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan

The molecular mechanism for regulation of the genes involved in the biosynthesis of amino acids is poorly identified in *Thermus thermophilus*. In this study, we analyzed the transcriptional control of the major lysine biosynthetic gene cluster in *T. thermophilus*. S1 nuclease mapping revealed that the transcription, which is repressed by lysine, starts at 111 bp, upstream of the translational start codon, ATG, for the homocitrate synthase (hcs) gene. The 5’-leader region of 111 bp carries a sequence that can encode a short peptide of 14 amino acids with tandem-arranged lysine residues in its sequence. The nucleotide sequence of the region suggests that the transcript can form complicated secondary structures. Deletion of most of the 5’-leader region or mutation of the tandem lysine codons suppressed the transcriptional repression by lysine. Mutation of the tandem codons from lysine to glutamine resulted in glutamine-dependent repression of the gene connected downstream, indicating that the leader peptide mediated the transcriptional attenuation of the gene expression. This is the first report demonstrating the transcriptional regulation of amino acid biosynthesis in *T. thermophilus*. Two pathways are known for lysine biosynthesis: the diaminopimelate pathway found in most bacteria and plants, and the α-aminoacidipate (AAA) pathway found in yeast and fungi (1). In the former pathway, lysine is synthesized from aspartate via diaminopimelate, whereas lysine is synthesized from 2-oxoglutarate through AAA in the latter pathway. *Thermus thermophilus* is a thermoacidophilic bacterium that can grow maximally at 70°C. However, we found that *T. thermophilus* synthesizes lysine not via diaminopimelate but through AAA, which was the first discovery of a diaminopimelate-independent pathway for lysine biosynthesis in prokaryotes (2, 3). We cloned genes involved in lysine biosynthesis from *T. thermophilus* (2, 4–7) and elucidated the lysine biosynthetic pathway in *T. thermophilus*, in which the first half of the pathway (the synthesis from 2-oxoglutarate to α-aminoacidipate) is the same as that found in fungi and yeast, whereas the second half of the pathway (the conversion of α-aminoacidipate to lysine) proceeds in a manner similar to arginine biosynthesis (Fig. 1).

Depending on the availability of the amino acid, its biosynthesis is controlled by two different systems: inhibition of the enzyme responsible for the first step in the biosynthetic pathway in most cases, and repression of gene expression. In the lysine biosynthesis of *T. thermophilus*, we found that homocitrate synthase, which catalyzes the first reaction in the pathway, is regulated by lysine (8). However, the regulation of lysine biosynthesis in gene expression has not yet been analyzed. All enzymes in the lysine biosynthesis of *T. thermophilus* analyzed thus far were found to be able to catalyze the reactions for related metabolic pathways, such as reactions for the tricarboxylic acid cycle and arginine biosynthesis (9). It is therefore very interesting to analyze the mechanism for transcriptional regulation of the gene involved in the novel lysine biosynthesis found in *T. thermophilus*. As the first step to elucidate the regulation of lysine biosynthesis in this microorganism, we analyzed the transcriptional regulation of the major lysine biosynthetic gene cluster. The results indicate that gene expression is regulated through a mechanism similar to that known for the trp operon in *Escherichia coli* (10, 11). This is the first report demonstrating the regulatory mechanism of expression of genes involved in amino acid biosynthesis in *T. thermophilus*.

**MATERIALS AND METHODS**

**RNA Isolation and Analysis**—Total RNA was isolated from *T. thermophilus* HB27 cells grown in minimal medium containing 0.1 mM CuSO₄, 0.4 mM ZnSO₄, 4 mM MnCl₂, 0.6 mM Na₂MoO₄, 0.6 mM VO₃, 13 mM MgCl₂, 2.25 mM CaCl₂, 13 mM FeSO₄, 6 mM CoCl₂, 0.15 mM NiCl₂, 0.001% biotin, 0.0001% thiamine, and 0.0001% H₂SO₄ with or without various additives by RNase-free DNase I (Takara, Kyoto, Japan) and 10 pmol of synthetic oligonucleotide primers, each having the template sequence of a gene (Table I) (Genset, Kyoto, Japan), by using Thermoscript™ (Invitrogen) as a transcriptase, according to the manufacturer’s instructions. Two microliters of the reaction mixture were then mixed with the template primer and another primer with the non-template sequence of the template primer and another primer with the non-template sequence of an upstream gene. After the addition of Ex-Taq polymerase (Takara), thermal cycling (95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min) was repeated 20 times. Different PCRs were also performed with samples prepared without reverse transcription to check for DNA contamination in the samples.

For S1 nuclease mapping analysis, 40 μg of total RNA extracted from *T. thermophilus* cells grown in various conditions were mixed with a 32P-labeled DNA fragment containing the 360/180 region (the first nucleotide of the translational initiation codon of the hcs gene was indicated as +1) in 20 μl of hybridization buffer (3 mM sodium acetate, 0.4 mM NaCl, 1 mM EDTA, and 50 mM HEPES, pH 7.4) at 70 °C for 5 min, and the hybridization mixture was gradually (over a 12-h period) cooled to 45 °C. Three hundred microliters of S1 digestion buffer (2 mM NaCl, 20
OF1053GD, which lacks the endogenous construct plasmids were then introduced into hcsing position of pET26–5714/Nde-Not to substitute for the template with appropriate primers and introduced into the correspond-
a NotI-NdeI fragment by PCR using
-leader region of gene as a NotI-NdeI fragment. Each DNA fragment containing the pET26–5714/Nde-Not, carries the slpA NotI site upstream of the
formed to eliminate the internal NdeI site in
resulting plasmid, pET26–5714S, site-directed mutagenesis was per-
) (Novagen, Darmstadt, Germany). Using the re-
mM ZnSO4, and 0.3 M sodium acetate, pH 4.6) and 100 units of S1
stream of the hcs/H9004 hcs/AgαT
resulting plasmid, pET26–5714S, site-directed mutagenesis was per-
Enzymes responsi-
bles to the corresponding reactions in the triacylceramide acid cycle and arginine biosynthesis are shown as gene products in E. coli in most cases.

**Table 1**

| Name          | Nucleotide sequence |
|---------------|--------------------|
| hcs-lysT/Fw   | 5′-CTTACCTACGGGAGGAGGAC-3′ |
| hcs-lysT/Rv   | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysT-lysU/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysT-lysU/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysU-lysV/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysU-lysV/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysV-lysW/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysV-lysW/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysW-lysX/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysW-lysX/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysY-lysZ/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysY-lysZ/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |

**RESULTS**

**Determination of the Transcriptional Unit of Genes in a Major Lysine Biosynthetic Gene Cluster**—The major lysine biosynthetic gene cluster of *T. thermophilus* contains eight genes (hcs, lysT, lysU, lysV, lysW, lysX, lysY, and lysZ), which are arranged in the same direction, and it is therefore suggested that these genes are transcribed as a single mRNA. To verify this possibility, RT-PCR analysis was performed with total RNA isolated

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stream of the hcs/H9004 hcs/AgαT
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| lysT-lysU/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysU-lysV/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysU-lysV/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysV-lysW/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysV-lysW/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysW-lysX/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysW-lysX/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysY-lysZ/Fw  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |
| lysY-lysZ/Rv  | 5′-GGGGAGGCAGGCTTGGCGAG-3′ |

**RESULTS**

**Determination of the Transcriptional Unit of Genes in a Major Lysine Biosynthetic Gene Cluster**—The major lysine biosynthetic gene cluster of *T. thermophilus* contains eight genes (hcs, lysT, lysU, lysV, lysW, lysX, lysY, and lysZ), which are arranged in the same direction, and it is therefore suggested that these genes are transcribed as a single mRNA. To verify this possibility, RT-PCR analysis was performed with total RNA isolated
from cultures of *T. thermophilus* HB27 grown in minimal medium. As shown in Fig. 2, a DNA fragment was amplified for the connecting portion between every two genes when the samples were treated with reverse transcriptase. This suggested that all genes in the cluster form an operon, which is transcribed in a polycistronic manner, and therefore the expression of genes involved in lysine AAA biosynthesis is controlled by the promoter located in the upstream region of the hcs gene. Hereafter, we refer to this promoter as the hcs promoter.

**Determination of the Transcriptional Start Point of the hcs Gene**—To determine the transcriptional start point of the hcs promoter, we performed S1 nuclease mapping. From the analysis, the transcription was found to start from the adenine residue, 111 bp upstream of the translational initiation codon for hcs (Fig. 3, A and B). Upstream of the transcriptional start point, sequences TTGACA and TACCCCT, which are similar to those of −35 and −10, respectively, and therefore can be recognized by the principle σ factor of RNA polymerase, were found at appropriate positions with 17-bp spacing (Fig. 3B). When similar S1 nuclease mapping was conducted with total RNA prepared from *T. thermophilus* cells, which were cultured in the presence of 5 mM lysine, the transcription signal was dramatically decreased. Thus, it was demonstrated that the expression of the major lysine biosynthetic gene cluster is controlled by lysine.

**hcs Leader Region Is Required for Regulation by Lysine**—The region between the transcriptional start point and the hcs initiation codon, 111 bp in length, contains several palindromic sequences, suggesting that it can form complicated secondary structures. In this region, there is a short open reading frame capable of encoding a peptide of 14 amino acids that contains tandem-arranged lysine residues at the 6th and 7th positions. These features of the upstream region reminded us of the transcription regulation found in the amino acid biosynthetic genes in enteric bacteria (25). Hereafter, we refer to this 111-bp upstream region as the hcs leader region. We examined the function of the leader region in lysine-dependent transcriptional control of lysine biosynthesis. For this purpose, we constructed a convenient assay system with the agaA gene encoding a-galactosidase as the reporter (Fig. 4A). When plasmid pTThcs-plp, which carries the hcs promoter, leader region, and agaA gene from *B. stearothermophilus*, was introduced into *T. thermophilus* OF1053GD, which lacked the endogenous a-galactosidase gene (agaT), a-galactosidase activity was detected, which decreased in a dose-dependent manner for lysine, indicating that our assay system was functional for the analysis of transcriptional regulation. On the other hand, a-galactosidase activity was not affected by lysine when cells harboring pTThcs-pΔlp, which carries the hcs promoter and agaA gene but lacks the hcs leader region, were used. This indicated that the hcs leader region is important for the regulation of transcription by lysine.

**Mutations at Tandem-arranged Lysine Codons**—To further elucidate the mechanism of transcriptional control of the hcs promoter, we constructed two other plasmids, pTThcs[KK/KK]-plp and pTThcs[KQ/KQ]-plp, in which the tandem-arranged lysine codons, AAAAAA, in the putative leader peptide were replaced with AAGAAG and CAGCAG, respectively. The former was designed to direct the production of the same leader peptide with two lysine residues, and the latter was designed to produce a peptide with two glutamates at the corresponding positions. *T. thermophilus* harboring pTThcs[KK/KK]-plp gave an activity profile similar to that by *T. thermophilus* carrying pTThcs-plp (Fig. 4B). However, *Thermus* cells harboring pTThcs[KQ/KQ]-plp showed a-galactosidase activity that was independent of lysine addition but markedly decreased by glutamine (Fig. 4B). We also examined the effect of other amino acids, glutamate, alanine, arginine, and threonine, on a-galactosidase expression in *T. thermophilus* harboring pTThcs[KQ/KQ]-plp, and we found that the addition of these amino acids did not decrease a-galactosidase expression, and the only exception was obtained with glutamate. The addition of glutamate decreased expression of the agaA gene, although only to a small extent (data not shown), suggesting that its addition might increase glutamine concentration in cells. In any case, these results indicate that gene expression in the cluster for lysine biosynthesis is regulated through the translation-coupling mechanism.

Recently, direct interaction of the 5′-untranslated region of mRNA with lysine was shown to be responsible for transcriptional regulation in the expression of downstream genes in *B. subtilis*: this system is known as riboswitch (26–38). Our results are consistent with the idea that the hcs promoter is regulated through a “classical” attenuation mechanism, based on ribosome stalling, that affects mRNA secondary structures, as in the amino acid biosynthetic gene regulation of enteric bacteria, but not through a riboswitch-like system. To further support this hypothesis, we examined the effect of 6-amino-caproate, a structural analog of lysine, on the expression of a-galactosidase activity (Fig. 4B) and found no effect on hcs promoter activity by addition of the lysine analog. Considering that aminocycl 1 RNA synthetase discriminates its cognate amino acid from others distinctly, this observation also suggests that a shortage of lysyl-tRNA1<sup>α</sup> in cells causes attenuation of the major lysine operon in *T. thermophilus*.

**Detection of the 5′-Leader Peptide of hcs**—All data presented indicate the presence of the leader peptide (hcs leader peptide) and its involvement in the attenuation control of lysine biosynthetic gene expression. The presence of a similar putative 5′-leader peptide has been suggested in regulation of amino acid biosynthesis, however, it has not been identified experimentally, except in a few cases (39). Furthermore, the mRNA for the lysine biosynthetic genes starts from a position only 3 bp upstream of the putative translational initiation codon of hcs leader peptide, suggesting a Shine-Dalgarno sequence-independent ribosome recognition of the translational initiation site, which can be seen only in some cases (40). To verify the expression model for lysine biosynthetic genes, it is therefore necessary to prove the translation of the hcs leader peptide experimentally. For this purpose, we constructed plasmid pTThcs-plpf (Fig. 4A). This plasmid allowed *T. thermophilus* to produce hcs leader peptide-a-galactosidase (His<sub>8</sub>-tagged at
COOH terminus) fusion protein under the control of the hcs promoter. A crude extract of T. thermophilus cells harboring pThcs-plpf showed low but obvious α-galactosidase activity, which decreased in the culture in the presence of lysine (Fig. 4B), indicating that regulatory function is maintained even in the fusion construct. The fusion protein was purified by affinity chromatography using Ni\(^{2+}\) and Co\(^{2+}\) resins, as described under “Materials and Methods.” We also purified α-galactosidase with His\(_9\) tag at the COOH terminus in the same way, as a control. The molecular weight of a single subunit of His\(_9\)-tagged α-galactosidase is 84,000 whereas that of fusion protein is calculated to be 88,000. When the purified samples were
subjected to SDS-PAGE (Fig. 5), both proteins show the expected molecular masses on the gel, and the hcs leader peptide-a-galactosidase fusion protein migrated more slowly than non-fused protein, suggesting that the fused protein is translated from the ATG of the hcs leader peptide. For further confirmation, we determined the partial amino acid sequences of trypsin and lysylendopeptidase digest of the fused protein by mass spectrometry. Of 768 amino acid residues of the fused protein, 353 amino acid residues were assigned by the analysis. Among them, a sequence stretch, LGRRGVQPSKGFLSPGP-GSGRFSFSVAYNPCQKQFRLAGKASYVMQLFRSGYLAH-VYG, which corresponded to the 8th to 69th amino acid sequences of the fused protein, was included. The sequence contained 7 COOH-terminal amino acid residues of the hcs leader peptide, a linker region of 18 amino acid residues, and 37 successive amino acid residues from the Ser2 of AgaA. This result indicates that the hcs leader peptide is actually translated in T. thermophilus in a Shine-Dalgarno sequence-independent manner and functions in attenuation control of the expression of lysine biosynthetic genes.

DISCUSSION

In E. coli and B. subtilis, expression of the lysC gene, which encodes aspartate kinase responsible for the first reaction in the lysine biosynthetic pathway through diaminopimelate, is regulated by lysine. Cumulative studies coupled with comparative analysis of the 5’-untranslated region revealed a new mechanism of transcriptional attenuation (31–33, 38), known as riboswitch, because the 5’-region of transcript has the ability to sense an effector molecule directly, thereby affecting the fate of the transcript complex. Similar regulatory mechanisms have recently been found for other various biosyntheses, such as thiamine (26, 39), flavin (26, 28, 29), cobalamin (26, 27, 29, 35), and purines (26, 29). As shown in this study, the hcs leader region is responsible for regulation of the expression of the lysine biosynthetic genes in T. thermophilus. Therefore, a similar system might be deduced to control lysine biosynthesis in T. thermophilus. However, the hcs leader region is only 111 bp in length, which is shorter than the region for riboswitch (184–315 bp) and has no sequence similarity to that of lysC containing the L-box for sensing lysine. In place of riboswitch, we demonstrated that the hcs leader peptide is actually produced and involved in the attenuation control of lysine biosynthetic gene expression in T. thermophilus. Thus, the regulatory mechanism for lysine biosynthetic gene expression in T. thermophilus is different from the other microorganisms investigated thus far, although the mechanisms are similar in that leader regions play a critical role in the regulatory mechanism of gene expression.

The transcription-regulatory mechanism of lysine AAA biosynthesis elucidated seems similar to those of amino acid biosynthesis, such as trp, his, leu, thr, pheA, ilvGMEDA, and ilvBN in enteric bacteria (25). Based on the results of the reporter gene assay and referring to information on transcription attenuation accumulated in enteric bacteria, we propose that the hcs leader region has alternative structures depending on the availability of lysine in the environment (Fig. 6). RNA polymerase initiating transcription pauses somewhere downstream of the stop codon of hcs leader peptide. We assume that the upstream stem loop structure shown in Fig. 6A might be a possible candidate for the pause hairpin. Once a ribosome reaches the paused polymerase in the course of translation, the paused polymerase is released and re-initiates RNA synthesis. In a lysine-rich environment, in which the supply of charged lysine-charged tRNAlys is abundant, the translating ribosome pauses downstream of the stop codon of the hcs leader peptide. Under such conditions, sequence A in mRNA forms a base pairing with downstream sequence B (Fig. 6A). The resulting stem-loop structure followed by poly(U) extension is a structure typical of the p-independent transcriptional terminator. Therefore, elongation of the transcript toward the hcs structural gene is prevented. On the other hand, under conditions of lysine starvation, in which the level of lysine-charged tRNAlys is low, the ribosome stalls at the tandem-arranged lysine codons. Transcription then proceeds, and the mRNA folds into an alternative stable form in which base pairing between sequences A and B necessary for forming the terminator structure is prevented, allowing the transcript to elongate toward downstream genes (Fig. 6B).

In the reporter assay, 6-aminocaproate, which is a lysine

![Fig. 5. SDS-PAGE of purified hcs leader peptide-a-galactosidase fusion protein.](image)

![Fig. 6. Proposed mechanism for attenuation control of the major lysine biosynthetic gene cluster.](image)
analog lacking α-amino group, did not affect the transcription activity at all. This contrasts with the \textit{lysC} promoter of \textit{B. subtilis}, which responds to S-aminoethylcysteine, a lysine analog, through the L-box, although it is 10-fold less effective than lysine in promoting transcription termination (33). This may reflect the difference in the regulatory mechanism for lysine biosynthesis between \textit{T. thermophilus} and \textit{B. subtilis} depending on the concentrations of lysine-tRNA\textsubscript{lys} and effector itself, respectively. We also tried to examine the effect of S-aminoethylcysteine on the \textit{hcs} promoter. However, because \textit{T. thermophilus} is very sensitive to S-aminoethylcysteine for growth, we could not add the compound at effective concentrations (data not shown). However, it is now clear that the regulatory mechanism for gene expression is completely different in lysine biosynthesis, although both are responsible for the biosynthesis of the same amino acid.

The expression of biosynthetic genes for primary metabolism is severely regulated by an effector molecule, the product in most cases. In the \textit{trp} operon in \textit{E. coli}, which possesses a regulatory mechanism similar to the lysine biosynthesis of \textit{T. thermophilus}, a repressor protein encoded by \textit{TrpR} is the primary factor controlling gene expression in tryptophan biosynthesis. Therefore, it could be considered that attenuation found in the \textit{trp} operon is the secondary factor controlling the gene expression. In this sense, the attenuation control of the \textit{trp} operon in \textit{E. coli} serves as a minor tuner for the whole system controlling tryptophan biosynthesis. In lysine biosynthesis in \textit{T. thermophilus}, however, the effective concentration of lysine to control gene expression is quite high, as revealed by S1 nuclease mapping and the reporter assay. This suggests that leader peptide-mediated transcriptional attenuation may be only a single mechanism for regulating the expression of the major lysine biosynthetic operon in \textit{T. thermophilus}.

In this study, we have demonstrated the regulatory mechanism of the major lysine biosynthetic gene cluster in \textit{T. thermophilus}. In addition to the genes in the major gene cluster, lysine biosynthesis requires at least four genes, which encode homoisocitrate dehydrogenase, α-aminoadipate aminotransferase, N\textsubscript{2}-acetyllysine aminotransferase, and N\textsubscript{2}-acetyllysine deacetylase. Although the last two genes seem to be present in \textit{T. thermophilus}, although both are responsible for the biosynthesis of the same amino acid.

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