A conserved RNA structure (*thi* box) is involved in regulation of thiamin biosynthetic gene expression in bacteria

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The *thiCOGE* genes of *Rhizobium etli* code for enzymes involved in thiamin biosynthesis. These genes are transcribed with a 211-base untranslated leader that contains the *thi* box, a 38-base sequence highly conserved in the 5′ regions of thiamin biosynthetic and transport genes of Gram-positive and Gram-negative organisms. A deletion analysis of *thiC-lacZ* fusions revealed an unexpected relationship between the degree of repression shown by the deleted derivatives and the length of the *thiC* sequences present in the transcript. Three regions were found to be important for regulation: (*i*) the *thi* box sequence, which is absolutely necessary for high-level expression of *thiC*; (*ii*) the region immediately upstream to the translation start codon of *thiC*, which can be folded into a stem-loop structure that would mask the Shine-Dalgarno sequence; and (*iii*) the proximal part of the coding region of *thiC*, which was shown to contain a putative Rho-independent terminator. A comparative phylogenetic analysis revealed a possible folding of the *thi* box sequence into a hairpin structure composed of a hairpin loop, two helixes, and an interior loop. Our results show that thiamin regulation of gene expression involves a complex posttranscriptional mechanism and that the *thi* box RNA structure is indispensable for *thiCOGE* expression.

Thiamin pyrophosphate (TPP), also known as cocarboxylase, is the cofactor of key enzymes of carbon metabolism such as pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, transketolase, pyruvate decarboxylase, and others. TPP is made by the enzymatic coupling of two independently synthesized precursors, 4-amino-5-hydroxymethyl-2-methyl pyrimidine pyrophosphate and 5-(2-hydroxyethyl)-4-methyl thiazole monophosphate (1).

In *Escherichia coli* and *Salmonella enterica* serovar Typhimurium, several genes are known to participate in TPP biosynthesis (2). *thiC* and *thiD* are required for 4-amino-5-hydroxymethyl-2-methyl pyrimidine pyrophosphate synthesis (3, 4), whereas *dxs* (5, 6), *thiF*, *thiS*, *thiG*, *thiH*, and *thiI* are involved in 5-(2-hydroxyethyl)-4-methyl thiazole monophosphate synthesis (3, 7, 8). In addition, the product of the *thiE* gene couples 4-amino-5-hydroxymethyl-2-methyl pyrimidine pyrophosphate and 5-(2-hydroxyethyl)-4-methyl thiazole monophosphate to give thiamin monophosphate, which undergoes another phosphorylation catalyzed by the product of the *thiL* gene to form TPP, the biologically active form of vitamin B_1 (9, 10). A salvage enzyme (thiamin kinase) encoded by *thiK*, which incorporates exogenous thiamin into TPP (11), also exists. In both organisms the *thi* genes are located throughout the chromosome and are arranged in three operons and four single gene loci (2). *thiCEFSGH*, *thiMD* (ThiM and ThiD are involved in salvage of 5-(2-hydroxyethyl)-4-methyl thiazole and 4-amino-5-hydroxymethyl-2-methyl pyrimidine from the culture medium, respectively; ThiD also catalyzes the phosphorylation of 4-amino-5-hydroxymethyl-2-methyl pyrimidine monophosphate and *thiBPQ* (which code for an ABC type transport system for thiamin and TPP) are the operons transcriptionally regulated by TPP, whereas the single loci are not regulated (4, 12, 13). Although some point mutations have been isolated that affect the transcriptional regulation of *thi* genes, they map at min 10 on the *S. enterica* chromosome and at least one of them is an allele of *thiL* gene encoding thiamin monophosphate kinase, suggesting that TPP is the effector molecule involved in regulation of *thi* gene expression (10, 12). No regulatory genes have been identified so far in any bacterial species.

In *Rhizobium etli*, which nodulates and fixes nitrogen in a symbiosis with bean plants, we have characterized a cluster of four genes named *thiCOGE* whose products are involved in thiamin biosynthesis and that are presumably organized as an operon (14). *thiC*, *thiG*, and *thiE* are homologous to *E. coli* genes. *thiO* is a gene that codes for a predicted flavoprotein with homology to D-amino acid oxidases probably involved in 5-(2-hydroxyethyl)-4-methyl thiazole monophosphate production (14). The transcription start site (tss) of the *thiC* gene lies 211 bases upstream of *thiC* start codon. The RNA untranslated leader contains a 38-base sequence, named *thi* box, that is highly conserved in the upstream region of *thi* genes of organisms as diverse as *E. coli*, *Synechocystis*, *Bacillus subtilis*, and *Mycobacterium tuberculosis* (14).

In this work, we found by functional analysis of the untranslated RNA leader that repression by thiamin is a posttranscriptional event and that the *thi* box is an element indispensable for the expression of *thiCOGE*. We also show that the region surrounding the Shine-Dalgarno (S-D) sequence and the first 443 nt of the coding region participate in the regulation of *thiCOGE* expression by thiamin. We propose that the *thi* box is a conserved RNA regulatory element that exerts its function by folding into a secondary structure whose existence is supported by a covariational and phylogenetic analysis.

Materials and Methods

Bacterial Strains, Plasmids, and Culturing. *R. etli* strain CE3 was used for all of the experiments and was grown at 30°C in minimal medium (MM) with 10 mM NH_4Cl as the nitrogen source and 10 mM succinate as the carbon source or in peptone yeast extract medium as described (15). Thiamin hydrochloride (100 μg/ml) was added as indicated. Plasmids were conjugated into *R. etli* by biparental mating using the *E. coli* S17.1 strain previously transformed with the plasmid of interest (16). Culturing for studying the effect of added thiamin was done as follows: cells were grown to an OD_{600} of 0.6 in 100 ml of MM with or without thiamin as indicated. From this culture 30 ml were centrifuged and used to inoculate 50 ml of MM with or without thiamin. This culture was grown to an

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Abbreviations: TPP, thiamin pyrophosphate; tss, transcription start site; S-D, Shine-Dalgarno; MM, minimal medium.

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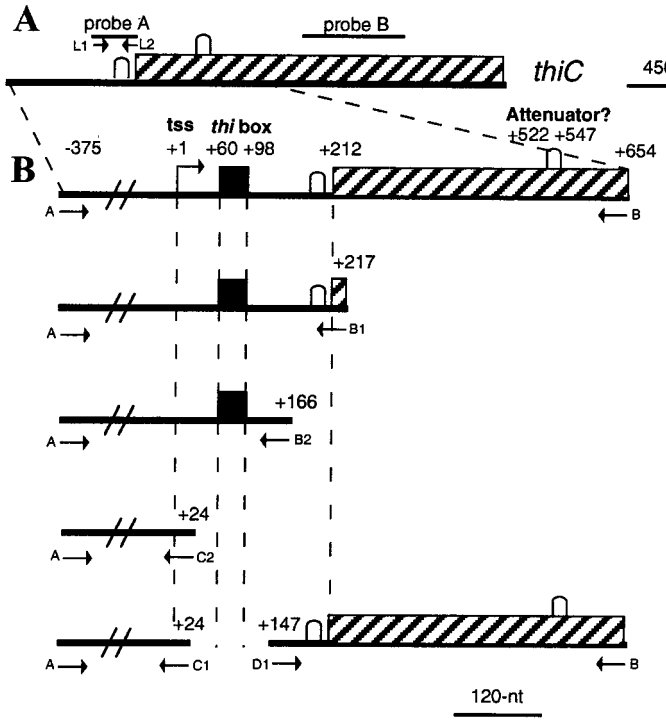

Construct	β-galactosidase activities THIAMIN —	+	RR
pMM7	573	29	19
pMM7.1	937	129	7.3
pMM7.2	687	869	1
pMM7.3	1,440	1,468	1
pMM7.4	21	25	1

Fig. 1. (*A*) Schematic map of *thiC* gene of *R. etli*. The stippled box represents the *thiC* coding sequence. The lines above represent the location of the probes used in the Northern experiment. (*B*) Deletion constructions fused to *lacZ*. The fragments obtained by PCR were cloned into pMP220 vector to generate *lacZ* transcriptional fusions for determination of β-galactosidase activities from cultures grown in MM with or without thiamin as indicated. The letters alongside the arrows represent the names of the oligonucleotides used to obtain the different PCR fragments. The nucleotide positions are given with reference to the tss (position +1) of *thiC*. The black box represents the *thi* box sequence, and the potential stem-loop structures in the transcript that comprises the S-D sequence and the attenuator are marked by an inverted U. β-galactosidase activity is expressed as nmol of *o*-nitrophenol produced min^{-1}·mg $protein^{-1}$. Representative results of three experiments are shown. RR is the repression ratio, the β-galactosidase activity observed in media without thiamin divided by the activity observed in media supplemented with thiamin.

OD_{600} of 0.7, and the cells were harvested for β-galactosidase measurements or RNA preparations.

Construction of *thiC-lacZ* Fusions. All of the deletion fragments were obtained by PCR amplification using plasmid pJMR01 as template (17). This plasmid contains an *Eco*RI fragment of 2.5 kb containing the regulatory region of *thiC* and 540 bp of *thiC* coding region. The amplified fragments were ligated to plasmids pBlueScript-SK (Stratagene) or pCAPs (18) and were sequenced on both strands. After purification and restriction, the fragments were ligated to vector pMP220 digested with *Bgl*II and *Xba*I or *Bgl*II and *Pst*I. The vector pMP220 contains the *E. coli lacZ* gene without a promoter generating transcriptional *lacZ* gene fusions (19). The following pairs of oligonucleotides were used in the amplification reactions (the sequences of all PCR primers are available on request): A-B, A-B1, A-B2, and A-C2 to construct plasmids pMM7, pMM7.1, pMM7.2, and pMM7.3, respectively. To obtain construct pMM7.4, two fragments were amplified by using oligonucleotides A-C1 and B-D1. First, the A-C1 fragment was cloned into pCAPS *Bgl*II–*Eco*RI. Then the B-D1 fragment was cloned into the recombinant clone carrying the A-C1 fragment in the *Eco*RI and *Xba*I sites. Finally, the *Bgl*II–*Xba*I fragment was excised, purified, and cloned into pMP220 digested with *Bgl*II–*Xba*I (Fig. 1*B*).

β-Galactosidase Measurements. The level of β-galactosidase was determined as described (20), by measuring the rate of hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside in cells permeabilized with SDS-$CHCl_3$. All assays were performed in triplicate.

RNA Slot-Blot Hybridization. Total bacterial RNA was isolated with the High Pure RNA Isolation Kit of Roche Molecular Biochemicals. Ten micrograms of RNA was slot-blotted into Hybond N^+ membranes, hybridized, washed, and exposed to film as described by the manufacturer (Amersham Pharmacia). Probe A was obtained by using oligonucleotides L1-L2 and plasmid pJMR01 as template in a PCR amplification reaction. Probe B is a 453-bp *Xho*I fragment derived from the central part of the *thiC* coding region contained in plasmid pJMR5 (from nucleotide +1044 to +1494) (14). The 16S probe is a 500-bp *Hin*dIII fragment containing the 16S rRNA gene from *E. coli* (21). All probes were radioactively labeled by using ^{32}P-dCTP and the random priming kit from Amersham Pharmacia.

Search of *thi* Box Sequences in GenBank. Homologs to the *thi* box sequence were searched by using the BLAST program as implemented in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) with the *R. etli thi* box as query (14). The GenBank entries of the sequences showing partial matches to the *thi* box were retrieved and were pairwise-aligned to the *R. etli thi* box by using the GAP program of the Wisconsin package (GCG). In all of the cases analyzed, thiamin biosynthetic genes were present downstream of the newly found *thi* box sequences.

Results

Location of the *thi* Box Sequence in the *E. coli* Genome. To determine the possible role of the *thi* box in gene expression, the *E. coli* genome was searched for the *thi* box sequence as detailed in *Materials and Methods*. Interestingly, the *thi* box sequence was found only in front of the three thiamin biosynthetic operons

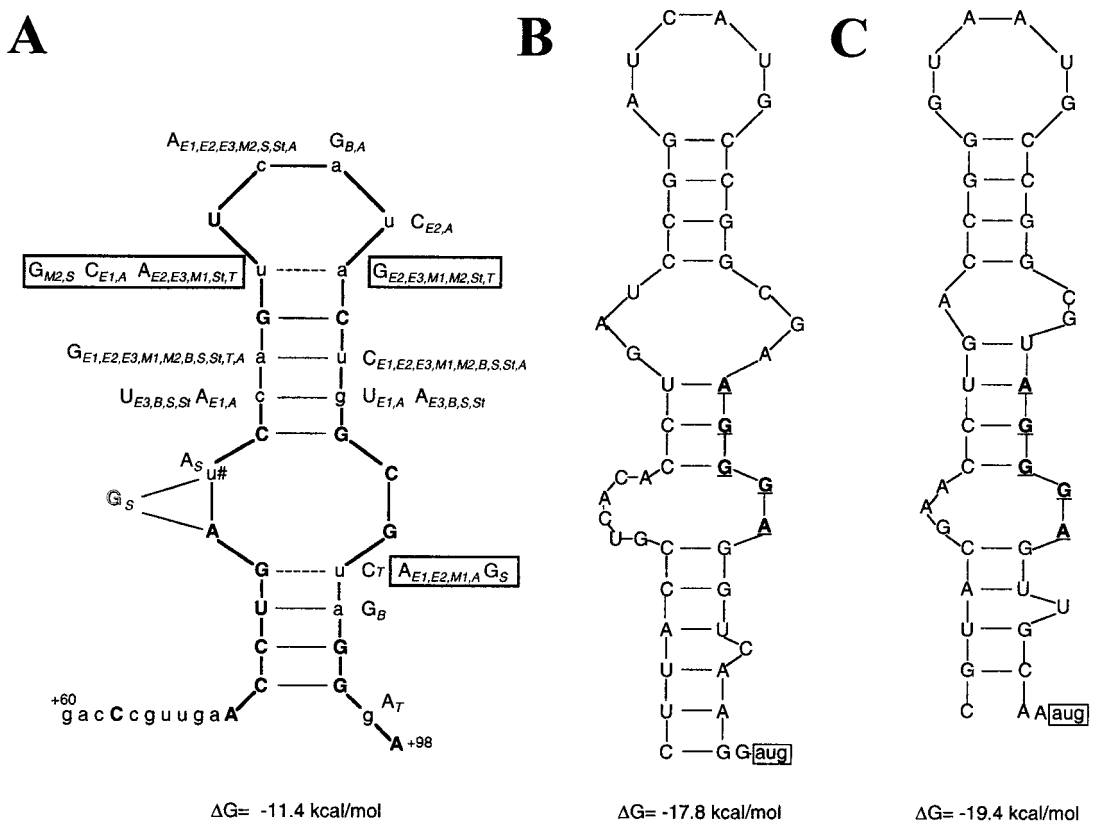

Fig. 2. (*A*) Proposed secondary structure for the *thi* box sequence. Capital bold letters represent conserved bases in all of the *thi* box sequences analyzed. The dashed lines represent hydrogen bonds that can be formed only in the *R. etli thi* box. The capital letters alongside the *R. etli thi* box represent the changes in sequence observed in the other *thi* box sequences. The boxed capital letters represent changes that would not permit the formation of base pairs. Subscripts represent the thi box sequences in front of genes: E1, E2, and E3, *thiC*, *thiB* and *thiM* genes of *E. coli*; M1 and M2, *thiO* and *thiC* genes of *M. tuberculosis*; B1, *thiA* gene of *B. subtilis*; S, *Synechocystis*; St, *Salmonella enterica*; T, *Thiocystis violacea*; A, *Aeromonas hydrophila*. The u marked with a # is absent in M2 *thi* box (see *C*). The G in outlined style is inserted between the As in S *thi* box. (*B* and *C*) Hairpin structures formed by the *thi* box sequences in front of the *thiO* and *thiC* genes of *M. tuberculosis*, respectively. The sequence AGGGA that is highly conserved in the 3′ end of the *thi* box sequences analyzed is represented in bold, underlined letters. The translation start codon of both genes is boxed.

(*thiCEFSGH*, *thiMD*, and *thiBPQ*), whose expression in *S. enterica* has been shown to be negatively regulated by TPP (4, 12, 13). This correlation suggests that the *thi* box is a regulatory element involved in repression of gene expression by thiamin.

Effect of Deletions of the *thiCOGE* Regulatory Region on Expression. As mentioned previously, an untranslated RNA leader of 211 nt containing the *thi* box is present in the 5′ region of the *thiCOGE* operon of *R. etli*. To determine whether the *thi* box and/or other sequences of this leader region participate in thiamin gene regulation, a series of transcriptional *lacZ* fusions to 3′-deletion derivatives were made.

Fig. 1*B* shows that the construct pMM7 with its fusion junction at +654 (all coordinates relative to *thiC* tss) exhibited 19-fold repression by thiamin. This construct contains 444 nt of the coding region of *thiC* encoding 148 aa of the N-terminal part of ThiC. The deletion derivative pMM7.1 with endpoint at +217 (carrying only 6 nt of the coding region of *thiC*) displayed a reduced degree of repression (repression ratio of 7.3). This result suggests that an element that plays a negative role in thiamin control lies in the region deleted (from +218 to +654).

3′ Deletion to +166 (pMM7.2) resulted in a complete loss of regulation. In this construct, the translation start codon of *thiC* is not present. This result suggests that in the region between +167 to +217 an element important in making *thiCOGE* expression repressible by thiamin is located.

Role of the *thi* Box Sequence. Further 3′ deletion of the untranslated leader removing the *thi* box resulted in high constitutive expression (pMM7.3, Fig. 1). In accordance with this result, a primer extension analysis of cells grown in the presence or absence of thiamin showed the same tss (data not shown). Therefore, to study the role of the *thi* box in gene expression, the effect of deletion of the *thi* box was examined in a *lacZ* fusion (construct pMM7.4, Fig. 1*B*). Removal of a region from +24 to +147 containing the *thi* box resulted in extremely low levels of expression. This result shows that the *thi* box is an element indispensable for *thiCOGE* expression in the presence of the rest of the *thiC* transcript.

***thi* Box Secondary Structure.** Previous work showed that the *R. etli thi* box is part of a 211-nt untranslated RNA leader (14). The tss of the three *E. coli* thiamin biosynthetic operons have not been mapped. However, in the three cases a −35,−10 promoter could be identified at around 200 bp upstream from the translation start codon, suggesting that the *thi* box sequence is part of long untranslated RNA leaders also in *E. coli*. As the function of many large single-stranded RNAs is thought to be determined, at least in part, by their structure, we investigated *in silico* whether the *thi* box sequence has the potential to form a hairpin structure by using the M-FOLD program (22, 23). A structure with a ΔG = −11.4 kcal/mol was found and is shown in Fig. 2*A*. The previously identified *thi* boxes from *E. coli*, *Bacillus subtilis*, *M.*

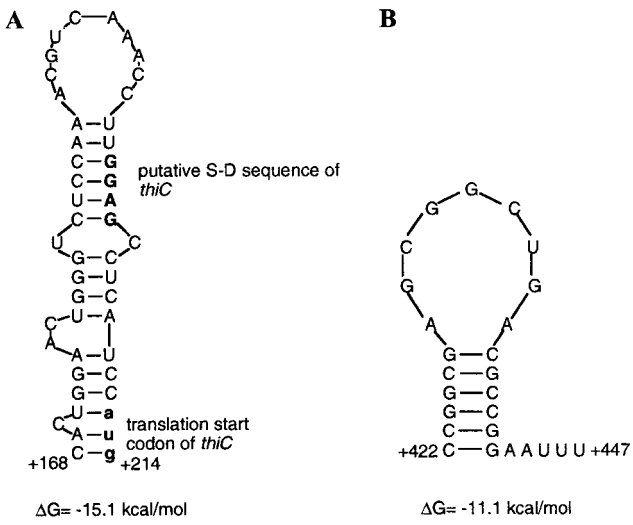

Fig. 3. (*A*) Proposed hairpin structure in the region surrounding the S-D sequence of the *thiC* gene of *R. etli*. The presumptive S-D sequence is represented by bold, capital letters and the translation start of *thiC* by bold, lowercase letters. (*B*) Putative Rho-independent terminator found in the coding region of *thiC*.

tuberculosis, *Synechocystis* sp., *S. enterica*, *Thiocystis violacea*, and *Aeromonas hydrophila* (14) also could form a similar structure. In fact, if one superimposes the structures thus generated, two important facts are revealed. The first is that of the 18 bases conserved in all of the *thi* box sequences analyzed, nine bases are part of the two helixes that can be formed (Fig. 2*A*). The second is that in the case of nonconserved bases that form part of the two helixes the changes in base composition on one side of a helix are compensated by matching changes in base composition on the opposite side of the same helix (Fig. 2*A*). These results strongly suggest that the putative hairpin structures identified are formed *in vivo* and are conserved in diverse bacterial species.

Previously we showed that in some organisms, like *M. tuberculosis*, the *thi* box is near the translation start codon whereas in other organisms, like *R. etli* or *E. coli*, the *thi* box is around 100 nt upstream. In Fig. 2 *B* and *C*, the hairpin structures formed by the *thi* box elements present in front of the *thiO* and *thiC* genes of *M. tuberculosis* are shown, respectively. The two presumptive S-D sequences are part of the *thi* box, which is located 7 and 8 nt upstream from the start codon of the *thiO* and *thiC* genes.

Other Putative RNA Secondary Structures Important for Regulation. The results obtained with the deletion derivatives suggested that the regions where the S-D sequence is located and the proximal part of the coding region of *thiC* are involved in regulation. Upon further analysis of both regions, two putative hairpin structures were found and are shown in Fig. 3. One putative structure (from nucleotide +168 to +214) could mask the S-D sequence (see Fig. 3*A*). The other structure present in the coding region of *thiC* (from nucleotide +522 to +547) has characteristics of a Rho-independent terminator (a stem formed by six G-C pairs followed by a run of two As and three Us at its 3′ end) (see Fig. 3*B*).

Expression Levels of *thiC* mRNA Leader and Coding Regions. To determine whether the putative attenuator found in the coding region of *thiC* is functional, a Northern experiment was performed with RNAs obtained from thiamin-grown or thiamin-deprived cells. We used three different probes, one that hybridizes to the untranslated leader sequence (probe A, from nucleotide +6 to +217), another derived from the central part

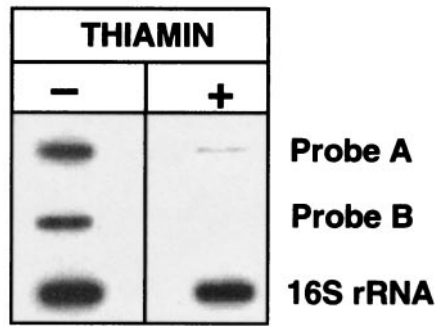

Fig. 4. *thiC* gene expression. RNA from the CE3 strain grown in MM with (+) or without (−) thiamin was slot-blotted and hybridized with probe A (a DNA fragment from nucleotide +6 to +217), probe B (an intra *thiC* fragment from nucleotide +1044 to +1494), and with the 16S rRNA gene of *E. coli*.

of the *thiC* gene (probe B, from nucleotide +1044 to +1494) (see Fig. 1*A*) and a third one derived from the 16S rRNA gene of *E. coli* that was used as loading control (21). With probes A and B, a signal was very evident in RNAs from thiamin-deprived cells. In contrast, a very faint signal was seen in RNAs from thiamin-grown cells only with probe A (see Fig. 4). The different pattern of hybridization observed with probes A and B suggests that the presumptive attenuator is functional.

Discussion

Although TPP is the cofactor of key enzymes of carbohydrate metabolism, little is known about the regulatory mechanisms involved in expression of its biosynthetic genes. Previous studies have shown that the *thiCOGE* operon of *R. etli* is transcribed with a 211-nt untranslated RNA leader (14). In this article we report a deletion analysis of reporter fusions to *thiC* that identified three regions of the transcript important for regulation by thiamin: the *thi* box located in the untranslated RNA leader, a second region located near the S-D site, and a third region located in the coding region of the *thiC* gene. In these three regions three putative RNA structures were identified *in silico*.

The *thi* box is highly conserved in the 5′ region of thiamin biosynthetic and transport genes from Gram-negative and Gram-positive organisms and cyanobacteria (14). Based on its evolutionary conservation and its presence in *E. coli* genes whose expression is regulated by TPP, it is thought to be involved in thiamin regulation. Further search for the *thi* box element in other bacterial genomes showed that it is present in *Thermotoga maritima*, *Streptomyces coelicolor*, *Mycobacterium leprae*, *Vibrio cholera*, *Sinorhizobium meliloti*, *Pseudomonas fluorescens* and in one species from Archae, *Thermoplasma volcanium* (GenBank accession nos. AE001747, AL133219, AL583918, AE004097, AF070520, AY007258, AP000994, respectively). These data show that the *thi* box sequence plays a fundamental role in an ancient mechanism regulating thiamin gene expression in bacteria. Phylogenetic analysis is the most accurate method for RNA secondary structure prediction. Some structures determined by this method include tRNAs (24), ribosomal RNAs (25–27), group I and group II introns (28, 29), RNAs from small nuclear ribonucleoproteins (30), hammerhead catalytic RNA (31), and RNase P RNAs (32). Further studies have confirmed these structures. A phylogenetic comparative analysis showed that all of the *thi* box sequences considered can be folded into a similar hairpin structure formed by two helixes, one interior loop, and a hairpin loop. From the 18 conserved bases in all of the *thi* box sequences analyzed, eight are involved in forming G-C pairs and two form an U-A pair (U-G in *B. subtilis thi* box) in the two helixes. One conserved base is present in the hairpin loop and four more lie in the interior loop. The other three conserved

bases are outside of the hairpin structure. It is possible that the eight conserved bases that do not form part of the helixes are specifically recognized by a regulatory factor. The other non-conserved bases that form part of the helixes show compensatory base changes that ensure base pair formation. The high conservation of sequence and structure of the *thi* boxes analyzed, the presence of the *thi* box in the 5′ region of thiamin biosynthetic and transport genes in different bacterial genomes, and the fact that the *thi* box associated with the *R. etli thiC* gene is transcribed and is absolutely necessary for *thiC* expression show that the *thi* box is a novel RNA regulatory element.

Concerning the second RNA structure identified *in silico*, a deletion with endpoint at +166 displayed unregulated high activities. This result suggests that in the region between +167 and +217 an element involved in repression of *thiC* expression by thiamin is present. A putative hairpin structure with a $\Delta G = -15.1$ kcal/mol was predicted in this region. This structure could mask the S-D sequence and the translation start codon of *thiC*. It is tempting to speculate that repression of *thiC* expression by thiamin depends on the formation of this structure. Mutagenesis of this structure will be important to determine its role in regulation. Interestingly, in *M. tuberculosis*, the first two RNA structures (the *thi* box and the hairpin structure) overlap (Fig. 2, *B* and *C*). In this bacterial species, the stabilization of the *thi* box structure in the presence of thiamin could occlude the ribosomal binding site, preventing translation of the *thiC* or *thiO* genes.

Finally, regarding the third RNA structure, deletion from nucleotides +218 to +654 revealed the existence of an element that plays a negative role in thiamin-dependent control. Such an element was found in the region from nucleotide +522 to +547. This sequence has the potential to form a hairpin structure with characteristics of a Rho-independent terminator. A Northern blot experiment using two probes, one derived from the leader region and the other from the central part of the coding sequence of *thiC*, downstream from the putative attenuator, showed that only the leader region-derived probe hybridized, giving a very faint signal, suggesting an effect on RNA stability. These results are consistent with the idea of a message halting at +547.

As mentioned previously, the polypurine sequence AGGGA, which is highly conserved in the 3′ end of the *thi* box sequences analyzed, is situated at a distance that would allow it to function as the S-D sequence of the *thiC* and *thiO* genes *of M. tuberculosis*. It is possible that all of the *thi* box sequences would be able to interact with the 30S ribosomal subunit. This interaction would be responsible for the protective effect of the *thi* box on *thiC* expression when the putative attenuator located in the coding region of *thiC* is also present. Examples of interactions between a S-D sequence and ribosomes leading to an increase in expression are documented for the genes *ermC* and *cry*IIIA of *Bacillus subtilis* and *Bacillus thuringiensis*, respectively. The expression of the *ermC* gene (which confers resistance to erythromycin) is increased upon addition of the antibiotic to the growth medium (33). The increase in expression results from the protection from degradation delivered by the ribosomes stalled on the *ermC* message (33). In *B. thuringiensis* it has been shown that a S-D sequence designated as STAB-SD is a determinant of stability of toxin *cry*IIIA mRNA (34). This STAB-SD is located 116 nt upstream of the start codon of the *cry*IIIA gene and is not involved in translation initiation (34). The STAB-SD is thought to enhance stability by interacting with the 30S ribosomal subunit.

A possible model of *thi* gene regulation by thiamin that can be drawn from the results reported here is shown in Fig. 5. As was stated before, transcription from the *thiC* promoter is constitutive. In the absence of thiamin, the *thi* box would not be folded and the sequence AGGGA could interact with the ribosomes. The interaction between the *thi* box and the ribosomes would impede the degradation of the message or, alternatively, the ribosomes so recruited by the *thi* box can be passed on to the S-D region where translation would start. The ribosomes translating the message would not allow the formation of the attenuator and transcription would proceed. In contrast, in the presence of TPP, the *thi* box would be folded into a hairpin structure and would not interact with the ribosomes, the hairpin structure in the S-D region will form and the ribosomes would not bind to the message. As a consequence, when transcription reaches the putative terminator, this sequence would be folded into a secondary structure and transcription would be prematurely terminated. The untranslated message then will be degraded.

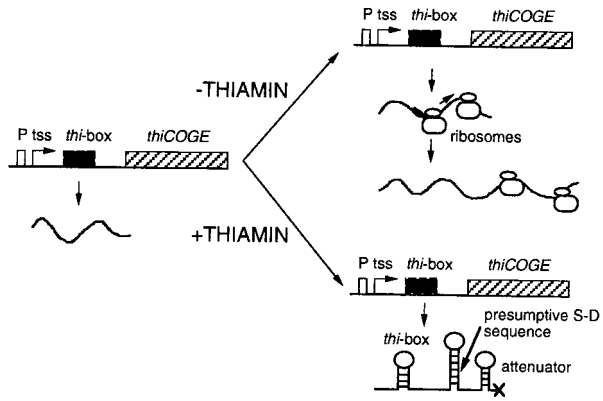

Fig. 5. Model for *R. etli thiCOGE* expression. (*Left*) The structural and functional organization of the regulatory and coding regions of the genes *thiCOGE* is represented. Transcription is constitutive from the *thiC* promoter. Depending on the availability of thiamin in the growth medium, this transcript will be elongated up to the end of the *thiE* gene (−thiamin condition) or would be prematurely terminated at the putative attenuator located at position +522 to +547 (+thiamin condition). The hairpin structures that can be formed by the *thi* box, the S-D sequence, and the putative attenuator are marked. *thiCOGE* genes are not drawn to scale. For details see the text.

In our model, a thiamin-dependent regulatory element could be involved in the interaction with the *thi* box RNA structure. However, no mutants affected in such a regulatory element have been isolated so far (10, 12). Also, titration of this putative regulator by the overexpression of the untranslated RNA leader failed to reveal its presence (data not shown). Thus, an interesting possibility is that in this case the direct binding of TPP to the *thi* box RNA structure could modulate the formation of the RNA structures identified in this work. Interactions between RNAs and small molecules like the one proposed here have been observed in the case of RNA aptamers that bind theophylline (35) and chloramphenicol (36) with very high affinity and specificity.

Regulation of *thiCOGE* expression shows striking similarities to repression of expression of the *btuB* gene and the *cob* operon by vitamin B_{12} or cyanocobalamin (37–39). In *E. coli* and *S. enterica*, these genes code for an outer membrane cobalamin transport protein and cobalamin biosynthetic enzymes, respectively. These genes are transcribed with unusually long leaders of 241 nt (*btuB*) or 468 nt (*cob* operon). Several mutations that led to decreased repression have been mapped to these leaders (40–42). The B_{12} box, a 25-nt element that is conserved in the leaders of the *btuB* and *cbiA* genes, is required for the expression of *btuB* (41, 43), as the *thi* box is required for thiamin-regulated expression of *thi* genes. In addition, a potential Rho-independent terminator structure that could serve as a transcriptional attenuator lies in the proximal portion of the coding sequence of *btuB*, as in the case of *thiC* (43). The primary control of *btuB* expression by adenosyl-cobalamin (the effector molecule) is exerted at the level of translation initiation where a hairpin structure sequesters the S-D sequence (44, 45). Similar to the

case for thiamin modulation of gene expression, no trans-acting factors involved in cobalamin repression have been found (46). Recently, it has been demonstrated that adenosyl-cobalamin inhibits the binding of 30S ribosomal subunits to *btuB* RNA and that a ribosome-associated, RNase-sensitive factor is needed to unfold the hairpin structure that masks the S-D sequence to allow ribosome binding (47). Furthermore, a cis-acting translational enhancer located upstream of the S-D sequence of the *cob* mRNA of *S. typhimurium* is required to unfold the inhibitory ribosome binding site hairpin in the absence of cobalamin (48). Also, it was shown that the untranslated leader of the *cob* mRNA is highly structured and that secondary and tertiary folding is required for normal repression by cobalamin (48). Other vitamin biosynthetic genes that seem to be regulated by a similar mechanism are the riboflavin genes, for which a conserved RNA structure element has been identified (49). Therefore, regulatory mechanisms similar to the one proposed here for thiamin could operate in the regulation of expression of other vitamin biosynthesis genes in bacteria.

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