A Covariant Change of the Two Highly Conserved Bases in the GTPase-associated Center of 28 S rRNA in Silkworms and Other Moths*

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The GTPase-associated center in 23/28 S rRNA is one of the most conserved functional domains throughout all organisms. We detected a unique sequence of this domain in Bombyx mori species in which the bases at positions 1094 and 1098 (numbering from Escherichia coli 23 S RNA) are C and G instead of the otherwise universally conserved bases U and A, respectively. These changes were also observed in four other species of moths, but not in organisms other than the moths. Characteristics of the B. mori rRNA domain were investigated by native polyacrylamide gel electrophoresis using RNA fragments containing residues 1030–1128. Although two bands of protein-free RNA appeared on gel, they shifted to a single band when bound to Bombyx ribosomal proteins Bm-L12 and Bm-P complex, equivalent to E. coli L11 and L8, respectively. Bombyx RNA showed lower binding capacity than rat RNA for the ribosomal proteins and anti-28 S autoantibody, specific for a folded structure of the eukaryotic GTPase-associated domain. When the C1094/G1098 bases in Bombyx RNA were replaced by the conserved UA bases, the protein-free RNA migrated as a single band, and the complex formation with Bm-L12, Bm-P complex, and anti-28 S autoantibody was comparable to that of rat RNA. The results suggest that the GTPase-associated domain of moth-type insects has a labile structural feature that is caused by an unusual covariant change of the U1094/A1098 bases to C/G.

23/28 S rRNA (23 S-like RNA) contains highly conserved domains that play fundamental roles in translation, i.e. the peptidyltransferase center, GTPase-associated domain, and sarcin/ricin target domain (1). The GTPase-associated RNA domain has been identified as the target for the antibiotic thiostrepton (2–4), which inhibits multiple turnover of elongation factor G (EF-G)-dependent GTP hydrolysis on eubacterial ribosomes (5). In Escherichia coli, this domain of 23 S RNA comprising residues 1051–1108, together with the saccin/ricin loop around residue 2660, has been shown to be part of the ribosome structure that interacts with EF-G (6, 7). This domain therefore seems to play a crucial GTPase-related role, probably in EF-G-dependent translocation of mRNA and tRNAs bound to ribosomal A and P sites. The tertiary structure of this RNA domain is recognized and stabilized by ribosomal protein L11 (8, 9). Recently, the folded structure of the RNA-L11 complex from E. coli and Thermotoga maritima has been solved by x-ray crystallography at 2.8- and 2.6-A resolutions, respectively (10, 11).

There is evidence for remarkable functional conservation of the GTPase-associated RNA domain between prokaryotes and eukaryotes: (a) the domain is interchangeable between yeast and E. coli ribosomes without significant loss of ribosomal function (12, 13); (b) the E. coli protein L11 cross-binds to the equivalent GTPase-associated domain of eukaryotic 28 S rRNA (14), and the rat homologue L12 also binds to the prokaryotic RNA domain (15); and (c) eukaryotic EF-G, a counterpart of EF-G, footprints position 1067 (E. coli numbering) within this domain of 28 S rRNA (16), which corresponds to one of the sites protected by E. coli EF-G (6). Comparative sequence analysis indicates that these similarities can be attributed to the extreme conservation of both primary and secondary structures of this RNA domain (17, 18). Of the 58 nucleotides in region 1051–1108, 24 are conserved in eubacteria, archaebacteria, eukaryotes, and chloroplasts (>85% of the species examined). Moreover, the 12 nucleotides in this region are conserved universally to include also mitochondria (see Fig. 1). Some of these conserved bases participate in the three-dimensional folding of the domain (10, 11), implying that the folded structure of the GTPase-associated domain is preserved in all organisms and is essential for its function.

Covariant base changes detected by comparative sequence analysis of rRNA have provided information on secondary structures and potential interactions, e.g. the base pair between residues 1082 and 1086 (19) and the base triple interaction between positions 1092/1099 and 1072 in the GTPase-associated domain (20). There is a limitation of this approach for functionally important bases since little sequence divergence has yet been detected. Despite the numerous and expanding sequence data on 23 S-like rRNAs (17, 18), only limited data are available for insect rRNAs. These include complete sequences of Drosophila melanogaster (21) and Aedes albopictus (22) and partial sequence, lacking the region of the GTPase-
associated domain, of Bombyx mori (25). Because of the huge number of insect species, these sequences are expected to provide substantial information on the sequence divergence of rRNAs. In this report, we present the first example that the two bases U1094 and A1098 among the universally conserved 12 nucleotides in the GTPase-associated RNA domain are changed to C and G, respectively, in silkworm species and other moths. To address the functional significance of this covariant change, we examined the effects of the base substitutions at positions 1094 and 1098 on the binding of ribosomal proteins and anti-28 S autoantibody (16, 24), which recognizes the tertiary structure of the RNA domain, and we discuss a structural feature of the RNA domain in moth-type insects caused by the covariant base change. A potential interaction between positions 1094 and 1098 is also discussed.

MATERIALS AND METHODS

Ribosomes, rRNA, and Ribosomal Proteins—Moth membrane-free ribosomes were prepared from the fat bodies or the posterior part of silk glands of last-instar larvae according to the procedure of Madjar and Fournier (25). The moths used were the silkworm B. mori (four strains: Daizo, E7, C124, and C124), the Chinese oak silkworm A. pernyi, the Japanese oak silkworm A. yamamai, the sweet potato hornworm Agrius convolvuli, and the common cutworm Spodoptera litura. Ribosomes were also prepared from two other insects by the method used for moths: from the fat bodies of imagos of the grasshopper Locusta migratoria and from whole bodies of third-instar larvae of the fly Musca domestica. The ribosomes samples were treated with pronase in the presence of 0.5 mM KCl (36) and recovered by ultracentrifugation as described previously (27). The rRNAs were phenol-extracted from the ribosomes of all species and used as the templates for sequencing. The ribosomal proteins equivalent to E. coli L11 and L8 complex were prepared from B. mori ribosomes according to methods applied for the rat counterparts, L12 and F complex (P protein complex corresponding of eukaryotic ribosomal F0 and are designated Bm-L12 and Bm-P complex, respectively. The identity of the Bm-L12 protein was confirmed by amino acid sequencing of the N terminus of a protease V8-cleaved peptide in a protein sequence (Model G1005A, Hewlett-Packard Co.) and comparison with the sequence data for Bm-L12 cDNA in the Bombbyx expressed sequence tag data base. Ribosome (30). P proteins Bm-P0, Bm-P1, and Bm-P2 were confirmed by reactivity with anti-P protein monoclonal antibody (31). The formation of Bm-P complex comprising the three P proteins was ascertained by native polyacrylamide gel electrophoresis as described previously (29).

**Sequencing of the GTPase-associated Domain of Insect 28 S rRNAs—**

Each rRNA sample (3 pmol) was mixed with 10 pmol of the DNA primer in 10 mM Tris-HCl (pH 7.5) and a protein sample as indicated in the figure legends, the mixture was further incubated at 30 °C for 10 min. RNA-protein binding was examined by electrophoresis on 6% polyacrylamide gel (acrylamide/bisacrylamide ratio of 40:1) at 6.5 V/cm with a buffer system containing 5 mM MgCl₂, 50 mM KCl, and 50 mM Tris-HCl (pH 7.6). The fraction of RNA bound to protein was estimated as the ratio of the RNA-protein complex to input RNA using imaging plates and a Storm imaging analyzer (Model 860, Amersham Pharmacia Biotech).

**Assembly of 28 S rRNA Sequence—**

The cDNA library including many clones for various sites of rRNA was prepared using total RNAs from the fat bodies of fifth-instar larvae of B. mori as described previously (36), except that pUC18 was used as vector instead of M13mp18. Sequencing of the cDNAs was carried out in an automated DNA sequencer (Models 373S and 377, Applied Biosystems, Inc.). Assembling >50 cDNA clones (the sequence data are available in the Bombbyx expressed sequence tag data base described above) gave nearly the complete nucleotide sequence of 28 S rRNA.

**RESULTS**

The nearly complete sequence of B. mori 28 S rRNA was derived by assembling many sequences from the B. mori cDNA library. The secondary structure was constructed by superimposing its sequence onto a model of the consensus secondary structure of eukaryotic 23 S-like rRNAs (18). The Bombbyx 28 S rRNA shares the conserved core secondary structure (data not shown). A novel characteristic of the sequence appeared in one of the functional regions, termed the GTPase-associated domain. Two bases at positions 1094 and 1098 (E. coli numbering) in the same helix were C and G, respectively, instead of U and A, which are highly conserved (>95%) in all 23 S-like rRNAs throughout eukaryotes, archaeabacteria, eubacteria, chloroplasts, and mitochondria (Fig. I (17)). No such substitution of the highly conserved bases was present at positions other than 1094 and 1098 in the 28 S rRNA sequence.

To confirm the unique sequence detected in the B. mori cDNA library, we analyzed rRNA extracted from the purified ribosomes. We prepared 80 S ribosomes from the silk glands of B. mori larvae (J107). The rRNA was phenol-extracted from the ribosomes and used as a template for sequencing by primer extension with reverse transcriptase (Fig. 2A). As expected, B. mori ribosome RNAs contained the highly conserved U and A bases, respectively, in the GTPase-associated domain (Fig. 2B). The same sequence was also observed in the other three strains of B. mori (Daizo, E7, and C124) (data not shown). It should be added that the isolated ribosomes carrying this unique sequence were active in poly(U)-dependent polyphenylalanine synthesis as well as EF-2-dependent GTP hydrolysis, and the activities were comparable to those of rat liver ribosomes carrying the conserved U1094 and A1098 bases (Table I).
The GTPase-associated Domain of Silkworm 28 S rRNA

Comparison of EF-2-dependent GTPase and polyphenylalanine synthesis activities between B. mori and rat ribosomes

The GTPase activity was analyzed using 2.5 pmol of high salt/puro-
ymycin-treated ribosomes (27), 8 pmol of EF-2 isolated from pig liver (43), and 3 nmol of [γ-32P]GTP as described previously (24). Poly(U)-de-
pendent polyphenylalanine synthesis was performed at 37 °C for 10 min in 75 μl of solution containing 5 pmol of the ribosomes, 40 pmol of
[14C]Phe-tRNA (400 cpn/mol), 10 μg of poly(U), 0.3 mM GTP, 5 mM
MgCl₂, 100 mM KCl, 50 mM NH₄Cl, 0.2 mM diithiobis, and 50 mM
Tris-HCl (pH 7.5) in the presence of 400 μg of the S200 fraction from
the silk gland cell lysate. The values are means of three experiments.

<table>
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<th>Assay</th>
<th>Ribosome</th>
<th>Rat</th>
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<tr>
<td>GTP hydrolysis (pmol/min)</td>
<td>34.7 ± 3.3</td>
<td>28.5 ± 1.2</td>
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<tr>
<td>Poly-Phe synthesis (pmol)</td>
<td>28.4 ± 0.9</td>
<td>31.2 ± 1.0</td>
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To test whether the base changes at positions 1094 and 1098 are specific to B. mori or not, we also prepared rRNAs from six other insects, i.e. four other species of moths (Lepidoptera) and two other than moth, and examined the RNA sequences of the GTPase-associated domain by primer extension analysis. The results are summarized in Fig. 3, together with sequences from rat (28), D. melanogaster (21), and A. albopictus (22). The base changes to C1094 and G1098 were observed in all species belonging to the group of moths (Lepidoptera; the Chinese oak silkworm A. pernyi, the Japanese oak silkworm A. yamamai, the sweet potato hornworm A. convolvuli, and the common cut-
worm S. litura), whereas the grasshopper L. migratoria, the flies M. domestica and D. melanogaster, and the mosquito A. albopictus have the conserved U1094 and A1098 bases. At positions other than 1094 and 1098 in this domain, all the insects have bases identical to those in the rat sequence, except changes at the less conserved positions 1062, 1063, 1073, 1075, and 1076 in the three species (Fig. 3). It is noteworthy that two highly conserved bases at the same stem-loop are changed simultaneously in the moths.

Synthetic RNA fragments are known to mimic the local rRNA structure and have been frequently used for studying the GTPase-associated domain (7, 9–11, 16, 29). We have shown that a rat rRNA fragment corresponding to residues 1030–1128 in E. coli 23 S rRNA retains efficient binding affinities for rat ribosomal proteins L12 and P complex, counterparts of E. coli L11 and L8 complex, respectively (29). We therefore used fragments of this region in B. mori and rat 28 S rRNAs and examined the effect of the covariant base change in the moth-
type GTPase-associated domain. These domain fragments con-
tained the conserved region 1051–1108 and the divergent 5′-
flanking (21 residues) and 3′-flanking (20 residues) regions in
which there were eight additional base substitutions between
B. mori and rat (Fig. 4A). The B. mori RNA migrated as two separate bands under native polyacrylamide gel conditions (Fig. 4B, lane 1), irrespective of annealing the RNA at 65 or 90 °C in the presence of various concentrations of Mg²⁺ (5–50
mM) and 350 mM KCl (data not shown). This is not due to the presence of two kinds of transcripts because the transcript of the Bombyx RNA showed a single band under denaturing gel conditions with urea (Fig. 4D). Upon the respective replacements of C1094 and G1098 with the conserved U and A bases in the Bombyx RNA, the RNA changed to a single band on the native gel (Fig. 4B, lane 2), similar to the rat RNA (lane 2). The RNAs were tested for binding to anti-28 S autoantibody, which recognizes a tertiary structure of the eukaryotic GTPase-asso-
ciated domain stabilized by Mg²⁺ (16). The Bombyx RNA bound very weakly to the anti-28 S Fab fragment (Fig. 4C, lane 1), but base substitutions of U1094 and A1098 markedly enhanced this binding (lane 2). This binding was comparable to that of the rat RNA (Fig. 4C, lane 3).

We prepared B. mori ribosomal proteins Bm-L12 and Bm-P complex, the silkworm counterparts of E. coli L11 and L8, respectively, according to methods applied to the rat homologues (28, 29). The RNA fragment covering the Bombyx GTPase-associated domain was tested for binding to these ribosomal proteins by gel retardation assay (Fig. 5). Although the protein-free Bombyx RNA showed two smeared bands, the addi-
tion of Bm-L12 (Fig. 5, lane 2), Bm-P complex (lane 3), or both proteins (lane 4) shifted them to a single band for the RNA-protein complex. Ribosomal proteins appear to stabilize an otherwise labile structural feature caused by the unique bases C1094 and G1098. In Fig. 6, Bm-L12 binding (A) and Bm-P complex binding (B) of the Bombyx RNA (lanes 1–4) were compared with those of the rat RNA (lanes 9–12) and of the Bombyx RNA with C1094 and G1098 replaced with U and A, respectively (lanes 5–8). The binding capacity of the wild-type Bombyx RNA for the proteins was lower than that of the rat RNA. The fractions of the Bombyx RNA bound to Bm-L12 and Bm-P complex were 0.23 and 0.59, respectively, when the proteins were added in 3-fold excess over RNA (mol/mol), whereas the fractions of the rat RNA bound to the respective proteins were 0.49 and 0.92. Replacement of C1094 and G1098 with the conserved U and A bases caused higher protein binding capacity (Fig. 6, A and B, lanes 5–8); the fractions of the variant RNA bound to Bm-L12 and Bm-P complex (0.42 and 0.87, respect-
ively) were comparable to those of the rat RNA at the same protein/RNA molar ratio. The fraction of RNA-BmL12 complex was less than that of RNA-Bm-P protein complex in each RNA sample, suggesting that Bm-L12 binds to RNA less tightly and is labile during gel electrophoresis compared with Bm-P complex. The gel mobilities of the RNA-protein complexes (RNA-Bm-L12 (Fig. 6A) and RNA-Bm-P complex (Fig. 6B)) were very similar, irrespective of RNA species, suggesting no marked difference in the tertiary structure stabilized by the protein binding among the three RNAs.

We further examined the effect of base substitutions in var-
ious combinations at positions 1094 and 1098 (Fig. 7). All RNA fragments except that with the combination of the conserved U1094 and A1098 bases showed more than two bands on the native gel (Fig. 7A), suggesting that both bases (U1094 and A1098) are involved in RNA folding into a compact tertiary structure. Unexpectedly, the base substitution of C1094 to U alone (Fig. 7B, lane 5) or of G1098 to A alone (lane 6) caused efficient binding to Bm-L12. Therefore, both bases C1094 and G1098 are responsible for the low protein binding property of the Bombyx RNA (Fig. 7B, lane 1). No protein binding was detected in the variant containing G1094 and C1098 (Fig. 7B, lane 3).

**DISCUSSION**

The GTPase-associated domain in 23 S-like rRNA (region 1051–1108 in E. coli) contains 12 highly conserved bases (Fig. 1). This study demonstrates that two of these bases, U1094 and A1098, simultaneously change in B. mori to C and G, respectively. We also detected the same replacements in five species of moths, but not in four other insects (Fig. 3). Therefore, the base changes at positions 1094 and 1098 are likely to be a covariance, suggesting a close functional relationship between the two positions. The bases at positions 1094 and 1098 may provide new criteria for the classification of insects.

Loop 1093–1098 containing U1094 and A1098 is one of the very highly conserved structural units in rRNAs. NMR studies with 14 nucleotide-mers have shown that U1094 participates in a very sharp turn termed the “U-turn” and that A1098 interacts with G1093 with non-canonical hydrogen bonds (“sheared G-A pair”) and stabilizes hexaloop 1093–1098 (37). Current crystallographic studies have confirmed these structural features in eubacterial RNA-L11 complexes (10, 11). The present data indicate that the Bombyx RNA does not retain the typical U-turn and sheared G-A pair and that these conserved structural features are not necessarily required for ribosomal function. In fact, the silkworm ribosomes carrying C1094 and G1098 have
of Bm-L12 (A) or Bm-P complex (B) and analyzed by gel retardation. The 32P-labeled RNA fragment (5 pmol each) comprising residues 1051–1108 in eubacterial 23 S rRNA has been solved by x-ray crystallography (10, 11); the two main consisting of residues 1051–1108 in eubacterial 23 S rRNA and 1098 in the GTPase-associated domain. The two adjacent hairpin loops are involved in the binding of the antibiotic thiostrepton, an inhibitor of EF-G activity (4, 40), and such a structure is unstable in the anti-28 S autoantibody recognition. It is likely that a unique structure formed by hairpin loops 1066–1078 and 1093–1098 is responsible for the immunoreactivity, and such a structure is unstable in the Bombyx RNA.

The two adjacent hairpin loops are involved in the binding of the antibiotic thiostrepton, an inhibitor of EF-G activity (4, 40), and are likely also to interact with EF-G as deduced by chemical probing data (6, 41). Therefore, the functional significance of the unusual covariant base changes of U1094 and A1098 to C and G on protein binding seems to be due to a feature of the unstable tertiary structure caused by the base changes. The two smeared bands of the Bombyx RNA shift to one band upon binding Bm-L12 in the gel retardation assay (Fig. 6A). More clearly, the shift to one band is observed upon binding the eukaryotic stalk protein complex (Bm-P complex), which is a counterpart of the E. coli L8 complex (Fig. 6B). The results imply that Bm-P complex and Bm-L12 modulate the tertiary structure of the GTPase-associated domain. The binding of the Bm-P and Bm-L12 proteins may compensate for a defective effect of the unique bases C1094 and G1098 on RNA folding.

Anti-28 S autoantibody from an autoimmune patient has been used as a probe of the structure and function of the eukaryotic GTPase-associated domain (16, 24). This anti-28 S autoantibody recognizes the RNA domain stabilized with Mg2+ and protects against chemical modification of bases U1066, G1067, A1098, and A1098 (E. coli numbering) lying in the two hairpin loops (16). The very weak recognition by anti-28 S autoantibody of the Bombyx RNA and its strong binding by the base replacements of C1094 and G1098 with U and A on Bm-L12 binding (A) and Bm-P complex binding (B). The 32P-labeled RNA fragment (5 pmol each) comprising residues 1030–1128 of B. mori 28 S rRNA (2530 cpm/pmol of specific radioactivity; lanes 1–4), the same RNA fragment except with C1094 and G1098 replaced with U and A (2810 cpm/pmol; lanes 5–8), and the RNA fragment for the equivalent rat region (3600 cpm/pmol; lanes 9–12) were incubated without protein (lanes 1, 5, and 9) or with 5 pmol (lanes 2, 6, and 10), 10 pmol (lanes 3, 7, and 11), or 15 pmol (lanes 4, 8, and 12) of Bm-L12 (A) or Bm-P complex (B) and analyzed by gel retardation. Bm WT, wild-type B. mori RNA.

The labile structural feature of the Bombyx GTPase-associated domain is consistent with protein binding properties. The Bombyx RNA shows lower binding capacity for Bm-L12 and Bm-P complex compared with the rat RNA (Fig. 6). Both bases C1094 and G1098 (not either one alone) seem to be responsible for this property (Figs. 6 and 7). It has been established that the tertiary structure of the GTPase-associated domain is recognized and stabilized by the ribosomal proteins L11 in eubacteria (9) and L12 in eukaryotes (29). Nearly all of the contact sites for these proteins lie on helix 1057–1081 of the RNA domain (10, 11, 29, 39). Because the bases at positions 1094 and 1098 are unlikely to be the sites of direct contacts with the protein, the effect of the covariant change of U1094 and A1098 to C and G on protein binding seems to be due to a feature of the unstable tertiary structure caused by the base changes. The two smeared bands of the Bombyx RNA shift to one band upon binding Bm-L12 in the gel retardation assay (Fig. 6A). More clearly, the shift to one band is observed upon binding the eukaryotic stalk protein complex (Bm-P complex), which is a counterpart of the E. coli L8 complex (Fig. 6B). The results imply that Bm-P complex and Bm-L12 modulate the tertiary structure of the GTPase-associated domain. The binding of the Bm-P and Bm-L12 proteins may compensate for a defective effect of the unique bases C1094 and G1098 on RNA folding.

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The two adjacent hairpin loops are involved in the binding of the antibiotic thiostrepton, an inhibitor of EF-G activity (4, 40), and are likely also to interact with EF-G as deduced by chemical probing data (6, 41). Therefore, the functional significance of the unusual covariant base changes of U1094 and A1098 to C and G in one of the loop regions in moths is of interest. A simple

high activity in the synthesis of silk proteins in vivo (38) and in poly(U)-dependent polyphenylalanine synthesis and EF-2-dependent GTPase in vitro (Table I). Our data pose a question about the real roles of the bases at the conserved positions 1094 and 1098 in the GTPase-associated domain.

The folded tertiary structure of the GTPase-associated domain consisting of residues 1051–1108 in eubacterial 23 S rRNA has been solved by x-ray crystallography (10, 11); the two stem-loops (helices 1057–1081 and 1087–1102) associate in a parallel fashion so that the two hairpin loops are in very close proximity. This compact structure is stabilized by long-range tertiary interactions between the two stems, e.g. the U1060-A1088 base pair and the G1071-(G1091-C1100) and C1072-(C1092-G1099) base triples (see Fig. 1). The crystal data show no evidence that the bases at positions 1094 and 1098 are involved directly in the long-range tertiary interactions to stabilize the tertiary folding of this domain. However, our results show marked effects of base changes at these positions on native gel electrophoretic patterns; only the RNA fragment with the conserved U1094 and A1098 bases shows a clear single band, whereas the other RNAs, including the wild-type Bombyx RNA, migrate as two or more bands (Figs. 4B and 7A). The appearance of more than one band on the native gel seems to reflect the presence of conformational isomer(s) that fail to fold into the compact tertiary structure. Our results suggest that the bases at positions 1094 and 1098 participate indirectly in efficient folding of the RNA. It is more conceivable that the U-turn at position 1094 and the sheared G-A pair between positions 1093 and 1098 are required to stabilize helix 1093–1098, and this may be relevant to the stability of the neighboring C1072-(C1092-G1099) base triple.
explanation for the two base changes is that they are compensatory and involved in base pairing between positions 1094 and 1098, although there is no evidence supporting this pairing in crystallographic studies (10, 11), NMR data (37), or in the effect of exchange of bases between positions 1094 and 1098 (Fig. 7B). The high reactivity of N-1 of A1098 with dimethyl sulfate argues for providing...

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