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Conformationally Restricted Elongation Factor G Retains GTPase Activity but Is Inactive in Translocation on the Ribosome

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Summary

Elongation factor G (EF-G) from *Escherichia coli* is a large, five-domain GTPase that promotes tRNA translocation on the ribosome. Full activity requires GTP hydrolysis, suggesting that a conformational change of the factor is important for function. To restrict the intramolecular mobility, two cysteine residues were engineered into domains 1 and 5 of EF-G that spontaneously formed a disulfide cross-link. Cross-linked EF-G retained GTPase activity on the ribosome, whereas it was inactive in translocation as well as in turnover. Both activities were restored when the cross-link was reversed by reduction. These results strongly argue against a GTPase switch-type model of EF-G function and demonstrate that conformational mobility is an absolute requirement for EF-G function on the ribosome.

Introduction

Following peptide bond formation on the ribosome, peptidyl-tRNA is moved from the aminoacyl-tRNA site (A site) to the peptidyl-tRNA site (P site) in a reaction (translocation) that is promoted by elongation factor G (EF-G) and strongly accelerated by GTP hydrolysis (Rodnina et al., 1997). The molecular mechanism of translocation and, in particular, the role of conformational changes of EF-G for translocation catalysis are a matter of debate. The three-dimensional structures of EF-G from *Thermus thermophilus* in the GDP-bound (Czworkowski et al., 1994; Al-Karadaghi et al., 1996) and the nucleotide-free (Ævarsson et al., 1994) forms are very similar. Although the structure of the GTP-bound form is not known, small-angle X-ray scattering studies in solution indicate that it is similar to that of the GDP-bound form (Czworkowski and Moore, 1997).

Cryo-electron microscopic reconstructions of ribosome-EF-G complexes suggest that EF-G undergoes significant conformational changes during translocation (Agrawal et al., 1998, 1999; Stark et al., 2000). The main difference between these conformations appears to be the position of domains 4 and 5 relative to domains 1 and 2 (Agrawal et al., 1999; Stark et al., 2000). This is in line with the essential function of domain 4 of EF-G in translocation demonstrated previously (Rodnina et al., 1997, and references therein). It has been proposed that the interaction of domain 5 with domain 1, and changes of this interaction, may determine the overall conformation of EF-G, depending on whether GTP, GDP·P_i, or GDP is bound to domain 1 (Borowski et al., 1996; Wintermeyer and Rodnina, 2000).

In the GDP form of EF-G, domains 1 and 5 are in close proximity to each other (Figure 1A). In order to test whether there are functionally relevant conformational changes at the interface between domains 1 and 5, we set out to fix the arrangement of the two domains by introducing a cross-link and to test the functional properties of cross-linked EF-G. A disulfide cross-link between two suitably placed cysteine residues was chosen because it could be readily reversed by reduction. Such an approach for restricting intramolecular mobility was used successfully in several cases (Matsumura and Matthews, 1989; Chervitz and Falke, 1995; Tiebel et al., 1998; Zhang et al., 1999).

Results

Disulfide Cross-Link between Domains 1 and 5 of EF-G

Based on the extensive sequence homology of *E. coli* EF-G to the factor from *T. thermophilus*, the positions of cysteines to be introduced into domains 1 and 5 were designed on the basis of the crystal structure of the latter in the GDP-bound form. In order to avoid complications by the three cysteine residues present in native EF-G, the mutations were introduced into EF-G in which these residues had been replaced with other amino acids (Wilson and Noller, 1998). As depicted in Figure 1B, the steric arrangement of cysteine residues at positions 162 in domain 1 and 649 in domain 5 should allow the formation of a disulfide bond between the two thiol groups. In fact, in the double mutant, EF-G(G162C, T649C), the disulfide bond formed spontaneously, indicating that no significant distortion of the molecule was required.

Mutant EF-G(G162C, T649C) was expressed in *E. coli*, and cross-linked EF-G(G162C, T649C) (referred to as cross-linked EF-G hereafter) was purified by affinity chromatography, gel filtration, and hydrophobic interaction chromatography (Experimental Procedures). The disulfide cross-link was fully reversible upon reduction with 2-mercaptoethanol to yield non-cross-linked EF-G (Figure 1C). Changes of the electrophoretic mobility on denaturing gels caused by an intramolecular disulfide cross-link, as shown here for cross-linked EF-G, were reported for other proteins (Tiebel et al., 1998; Zhang et al., 1999). Cross-linked and non-cross-linked EF-G eluted at the same position upon analytical gel filtration on Superdex 200 (data not shown), demonstrating that the cross-link was intramolecular. The contamination with non-cross-linked factor of purified cross-linked factor was estimated to 3%–4% (Figure 1C).

Cross-Linked EF-G Hydrolyzes GTP but Is Inactive in Turnover on the Ribosome

In the presence of ribosomes, native EF-G hydrolyzes GTP in a rapid turnover reaction. Cross-linked EF-G was

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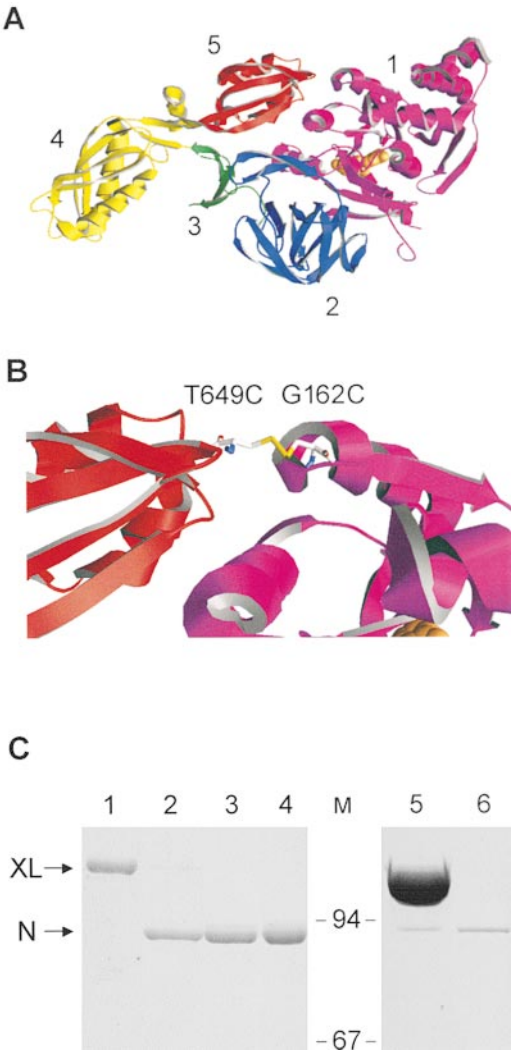

Figure 1. Disulfide Cross-Linking of Domains 1 and 5 of EF-G

(A) Crystal structure of EF-G·GDP from *T. thermophilus*. Domains are color coded: domain 1 (G domain), magenta; domain 2, blue; domain 3, green; domain 4, yellow; and domain 5, red. Bound GDP is depicted in space-filling mode.

(B) Close-up view of the domain 1-domain 5 interface. The disulfide cross-link between cysteine residues engineered into positions 162 and 649 (*E. coli* numbering; *T. th.*, 158 and 639) is indicated.

(C) Nonreducing gel electrophoresis of cross-linked and non-cross-linked EF-G. Lane 1, nonreduced, cross-linked EF-G (XL); lane 2, non-cross-linked EF-G (N) obtained by treatment with 2-mercaptoethanol (2-ME); lane 3, his-tagged EF-G lacking the three native cysteine residues; lane 4, same as lane 3, treated with 2-ME; lane 5, 10 μg cross-linked EF-G after purification; lane 6, 0.5 μg of non-cross-linked EF-G obtained by 2-ME treatment. The relative amount of non-cross-linked EF-G in lane 5 was estimated to 3%–4% by densitometry. M, positions of marker proteins (kDa).

virtually inactive in the reaction, whereas the activity was recovered upon reduction (Figure 2A). Ribosome titrations revealed k_{cat} = 4.6 ± 0.4 s^{-1} and K_M = 0.7 ± 0.3 μM for non-cross-linked EF-G, similar to the values determined for wild-type EF-G (data not shown). For the residual turnover, GTPase activity observed with cross-linked EF-G, k_{cat} = 0.2 ± 0.1 s^{-1}, and K_M = 0.5 ± 0.4 μM were found, that is, the values expected from the small

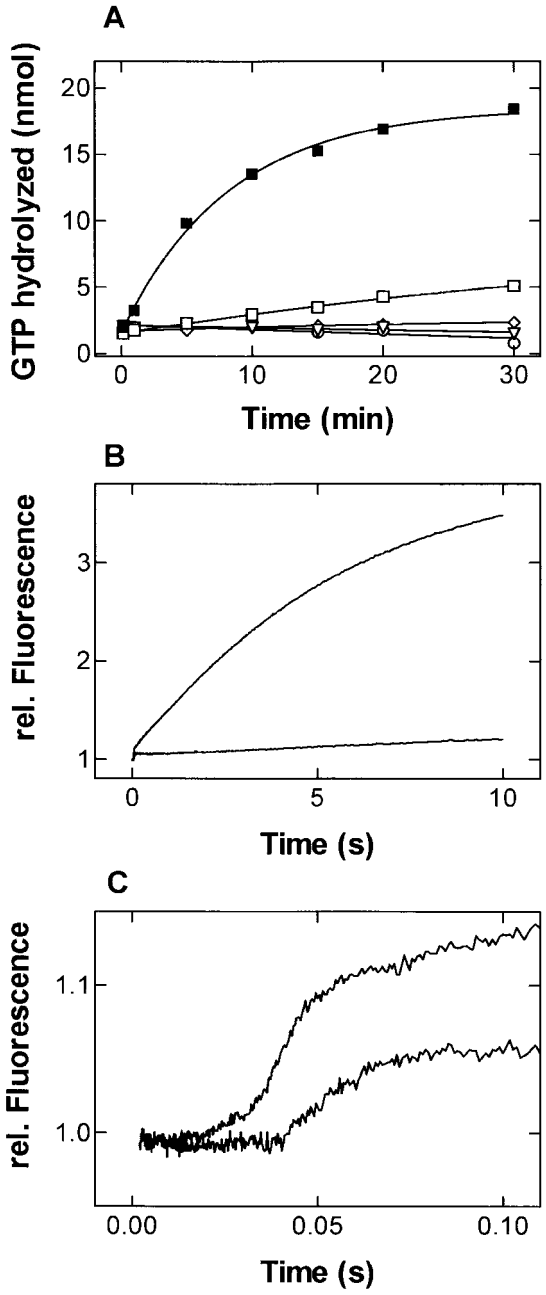

Figure 2. GTP Hydrolysis

(A) Turnover GTP hydrolysis. Cross-linked (open squares) and non-cross-linked (closed squares) EF-G (0.5 μM) was incubated with vacant ribosomes (0.5 μM) and [γ-^{32}P]GTP (1 mM) in buffer A at 37°C. The amount of liberated $^{32}P_i$ (50 μl samples) was determined by extraction (Experimental Procedures). Controls were performed with non-cross-linked EF-G alone (triangles), ribosomes alone (diamonds), and [γ-^{32}P]GTP alone (circles).

(B) P_i release, long time window. Cross-linked (lower trace) or non-cross-linked (upper trace) EF-G (final concentration, 0.4 μM) was rapidly mixed with vacant ribosomes (0.4 μM) in the presence of GTP (50 μM) in a stopped-flow apparatus at 37°C, and the fluorescence of labeled phosphate binding protein was monitored (Rodnina et al., 1999).

(C) P_i release, short time window of the experiment in (B). Approximate rates of P_i release from ribosome-EF-G complexes determined from the rapid fluorescence increase were 20 s^{-1}. Note the absence of the turnover phase with cross-linked EF-G.

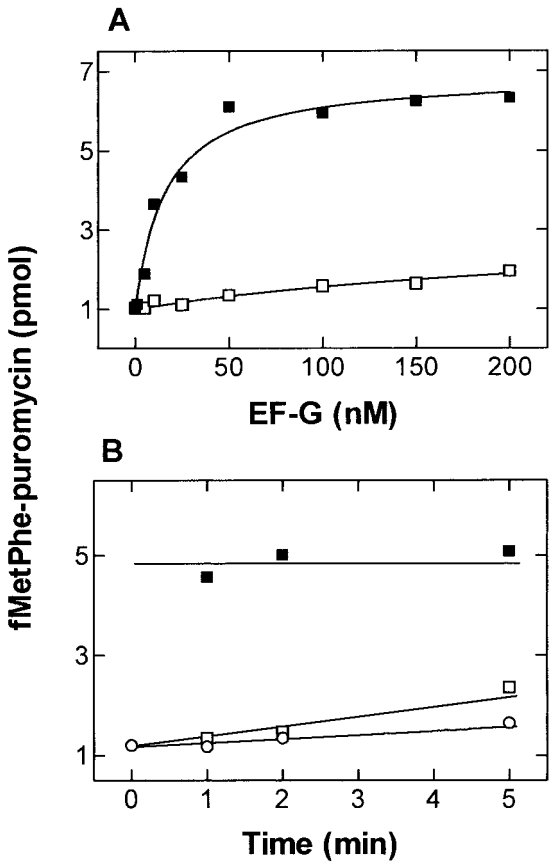

Figure 3. Translocation

(A) Turnover titration. Pretranslocation complex (7 pmol, 0.14 μM) was incubated with increasing amounts of cross-linked (open squares) and non-cross-linked (closed squares) EF-G in the presence of GTP (1 mM) in buffer A for 2 min at 37°C. Translocated f[^{3}H]Met-[^{14}C]Phe-tRNA^{Phe} was determined by reaction with puromycin (1 mM, 10 s, 37°C). Translocation was about 85% with wild-type EF-G (data not shown).

(B) Single-round translocation. Pretranslocation complex (0.2 μM), cross-linked (open squares) or non-cross-linked (closed squares) EF-G (0.6 μM), and GTP (1 mM) were incubated in buffer A at 37°C in the presence of fusidic acid (0.2 mM), which was added to minimize turnover. Open circles, control without EF-G.

amount of non-cross-linked EF-G present in the sample. Thus, cross-linked EF-G was virtually inactive in turnover GTP hydrolysis.

The same result was obtained when turnover GTP hydrolysis was followed by the release of inorganic phosphate, P_i, measured by the fluorescence increase of labeled phosphate binding protein (Figure 2B). This experiment also showed that cross-linked EF-G was about as active as non-cross-linked EF-G in single-round GTP hydrolysis (Figure 2C), although the extent of GTP hydrolysis was reduced to about 60%, indicating that part of the cross-linked protein was present in an inactive form. The conclusion from these results is that cross-linked EF-G was active in all steps up to and including P_i release, whereas it was inactive in turnover GTP hydrolysis.

The inhibition of turnover GTP hydrolysis may be due to the inability of cross-linked factor to dissociate from the ribosome after one round of GTP hydrolysis or, alternatively, due to the inhibition of GDP-GTP exchange

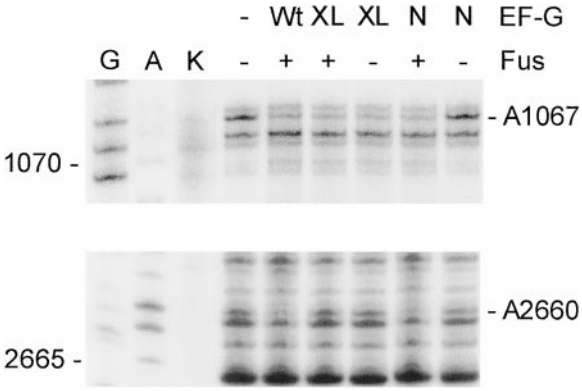

Figure 4. Dimethylsulfate Footprints of Cross-Linked EF-G on 23S rRNA

G and A, dideoxy sequencing lanes; K, control ribosomes without DMS; Wt, wild-type EF-G; XL, cross-linked EF-G; N, non-cross-linked EF-G obtained by 2-ME treatment. The presence or absence of fusidic acid (Fus) is indicated.

on cross-linked EF-G·GDP after dissociation from the ribosome. The latter possibility was excluded by an experiment in which the binding of [α-^{32}P]GDP to cross-linked factor, monitored by UV cross-linking and denaturing gel electrophoresis, was competed out completely by unlabeled GTP added in 20-fold excess over labeled GDP (data not shown). Thus, the inability of disulfide-cross-linked EF-G to perform GTP hydrolysis in turnover is attributed to the inhibition of dissociation from the ribosome after one round of GTP hydrolysis.

Cross-Linked EF-G Is Inactive in Translocation

When translocation was assayed at conditions of multiple turnover, non-cross-linked factor was highly active, whereas cross-linked factor exhibited a very low activity that, again, was attributed to contaminating non-cross-linked factor (Figure 3A). At single-round conditions, translocation was complete within 30 s with non-cross-linked EF-G (Figure 3B) and with wild-type EF-G (data not shown), in keeping with the high rate of translocation reported previously (Rodnina et al., 1997). In contrast, cross-linked EF-G was virtually inactive in single-round translocation (Figure 3B).

Chemical Footprints on 23S rRNA of Cross-Linked EF-G

Binding of EF-G to the ribosome results in characteristic protections of 23S rRNA against chemical modification by dimethyl sulfate (DMS) (Moazed et al., 1988). As shown in Figure 4, the non-cross-linked double mutant of EF-G behaved like wild-type EF-G in DMS footprinting, giving protections at residues 1067 (thiostrepton region) and 2660 (sarcin-ricin loop [SRL]) of 23S rRNA, but only when the ribosome-EF-G complex was stabilized by fusidic acid. Cross-linked EF-G gave the protections in the 1070 region, indicating that there was full binding, whereas it did not give the protections in the 2660 region. It is to be noted that the 1067 footprint of cross-linked EF-G was observed even in the absence of fusidic acid. This demonstrates that cross-linked EF-G is tightly bound to the ribosome, in agreement with the lack of turnover observed in the biochemical assays (Figures 2 and 3).

Previously, the SRL protections were not observed with EF-G mutants that lacked domains 4 and/or 5 and were strongly impaired in both translocation and turnover (Savelsbergh et al., 2000) or with wild-type EF-G when the two reactions were inhibited by thiostrepton (Rodnina et al., 1999). Thus, the absence of SRL protections is correlated with a strong inhibition of either translocation or turnover, indicating that the contact of EF-G with the SRL region takes place during or after translocation. Apparently, cross-linked EF-G does not reach this state.

Discussion

The observation that cross-linked EF-G binds to the ribosome and hydrolyzes GTP, but is inactive in translocation, demonstrates that intramolecular mobility at the interface between domains 1 and 5 is an absolute requirement for EF-G to promote translocation. Strikingly, the effect of cross-linking on the translocation activity of EF-G was much larger than the effects of deleting domains 1, 4, or 5 (Borowski et al., 1996; Rodnina et al., 1997; Savelsbergh et al., 2000) or of replacing GTP with GDP or with nonhydrolyzable GTP analogs (Rodnina et al., 1997, and references therein).

The activity of cross-linked EF-G in single-round GTP hydrolysis on the ribosome clearly shows that cross-linking of domains 1 and 5 did not prevent GTP binding, indicating that the cross-linked factor was able to undergo the conformational change from the GDP-bound form, which served to design the cross-link, to the GTP-bound form. This is in line with the observations that the two forms of the factor are not much different in overall structure (Czworkowski and Moore, 1997) and in binding to the ribosome (Baca et al., 1976; Rodnina et al., 1997). Therefore, the inactivity of cross-linked EF-G suggests that EF-G, in order to promote translocation, has to undergo more extensive conformational changes on the ribosome.

An idea how these conformations may look like is provided by three-dimensional reconstructions at 17–20 Å resolution of ribosome-EF-G complexes obtained by cryo-electron microscopy (Agrawal et al., 1998, 1999; Stark et al., 2000). According to these structures, during translocation there is a change at the interface of domain 5 with domain 1 together with a large movement of domain 4 relative to the body of EF-G (Stark et al., 2000). These intramolecular movements seem to be blocked, as are translocation and turnover, when domains 1 and 5 are cross-linked by the C162-C649 disulfide bridge. In conclusion, the present results are inconsistent with a GTPase switch-type model of EF-G function and rather support a model in which conformational changes of EF-G that are driven by GTP hydrolysis and/or P_i release are essential for factor function in translocation (Rodnina et al., 1997; Wintermeyer and Rodnina, 2000).

Experimental Procedures

Biochemical Assays

All experiments were carried out in buffer A (50 mM Tris-HCl [pH 7.5], 70 mM NH_4Cl, 30 mM KCl, and 7 mM $MgCl_2$) at 37°C. In assays with non-cross-linked EF-G, 2-mercaptoethanol ([2-ME] 10 mM) was added. Pretranslocation complexes were prepared with ribosomes from *E. coli* MRE 600 programmed with MFT-mRNA (Rodnina et al., 1999). GTP hydrolysis was analyzed either by extraction (Figure 2) or by thin layer chromatography (Michaelis-Menten titration, data not shown) as described previously (Rodnina et al., 1999).

P_i release was monitored by the fluorescence change of phosphate binding protein labeled with MDCC (N-[2-(1-maleimidyl)ethyl]-7-(diethylamino)coumarin-3-carboxamide [Molecular Probes]) (Brune et al., 1994) in a stopped-flow apparatus (Applied Photophysics) as described (Rodnina et al., 1999). To minimize phosphate contaminations, all solutions and the stopped-flow apparatus were preincubated with 0.1 mM 7-methylguanosine and 0.1 U/ml purine nucleoside phosphorylase (Brune et al., 1994).

Mutagenesis

The plasmid used for mutagenesis was provided by Kevin S. Wilson and Harry Noller, University of California, Santa Cruz. It contained the EF-G gene in which the three wild-type cysteine codons were substituted and that contained a sequence coding for a C-terminal affinity tag of six histidine residues (Wilson and Noller, 1998).

Mutagenesis was done according to the Quick Change method using Pfu polymerase (Stratagene). The following complementary primer pairs were used (mutated positions underlined): GGTTCGCGCACAGACGCGTTTTGATCTGG and CCAGATCAAAAC GCGTCTGTGCGCGAACC, to exchange glycin 162 for cysteine and to introduce a new MluI site; CGTGGATCTTGACGCCACAGACTT CAGATTCCTG and CAGGAATCTGAAGTCTGTGGCGTCAAGATC CACG, to exchange threonine 649 for cysteine and to introduce a new HinII site. Position 162 was mutated first, and that construct was used as template to obtain the EF-G(G162C/T649C) double mutant. Mutations were verified by DNA sequencing.

Expression and Purification of Cross-Linked EF-G

The plasmid coding for the double mutant EF-G(G162C/T649C) was transformed into *E. coli* BL21(DE3)pLysS cells by heat shock and the protein expressed for 4 hr after induction with IPTG. Pelleted cells were lysed by sonication in buffer B (50 mM Tris-HCl [pH 8.0], 100 mM KCl, 7 mM $MgCl_2$, 8 M urea, 10 mM 2-ME, and 0.1 mM PMSF). After centrifugation, a suspension of Ni^{2+}-NTA agarose (Qiagen) in buffer B was added to the supernatant. After 1 hr of agitation, the agarose was washed three times with buffer B and then by the same buffer at 6.8. The protein was refolded by stepwise removing urea and increasing pH of buffer to 7.5 and oxidized by removing 2-ME. The protein was eluted with 500 mM imidazole in buffer B without urea and 2-ME and concentrated by ultrafiltration in buffer A. Further purification was carried out by gel filtration on Superdex 200 (Pharmacia). Fractions containing cross-linked EF-G were identified by nonreducing polyacrylamide gel electrophoresis. Hydrophobic interaction chromatography was performed by Phenyl Superose FPLC (Pharmacia) after adding 0.85 M $(NH_4)_2SO_4$. The protein was eluted with a descending gradient of 0.85–0 M $(NH_4)_2SO_4$ in 50 mM Hepes-KOH (pH 7.5), desalted by FPLC on Mono Q (Pharmacia), and concentrated by ultrafiltration in buffer A. For storage at -80°C, 10% (v/v) glycerol was added. If not stated otherwise, cross-linked EF-G was reduced by incubation with 100 mM 2-ME in buffer A for 15 min at 37°C.

Dimethylsulfate Modification

The reaction with DMS was performed by incubating 0.4 μM ribosomes with 1.2 μM EF-G, cross-linked EF-G, or non-cross-linked EF-G (reduced with 2 mM dithiothreitol) in the presence of 1 mM GTP and, when present, 0.2 mM fusidic acid in 20 mM Hepes (pH 7.5), 30 mM potassium acetate, 70 mM ammonium acetate, and 7 mM magnesium acetate for 5 min at 37°C, followed by the addition of dimethyl sulfate and further incubation for 10 min at 37°C. Ribosomal RNA was isolated by phenol extraction. Methylated sites were determined by primer extension sequencing with AMV reverse transcriptase (Stern et al., 1988).

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