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Effects of Terminal Deletions in C5 Protein on Promoting RNase P Catalysis

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Deletion derivatives of C5 protein, the protein cofactor of *Escherichia coli* RNase P, were constructed as soluble MBP (maltose-binding protein) fusion proteins to assess the deletion effects on promoting RNase P catalysis and on binding to M1 RNA, the catalytic subunit of the enzyme. The C5 protein, with large terminal deletions, retained its promoting activity of RNase P catalysis under protein excess conditions *in vitro*. Some deletion derivatives complemented the temperature sensitive phenotype of *E. coli* A49 cells carrying the *rnp*A49 mutation. This ability also suggests that part of the C5 protein is enough to produce the catalytic activity of RNase P *in vivo*. Both the central conserved region, called the RNR motif, and the C-terminal region are essential for the binding of C5 protein to M1 RNA. Meanwhile, the N-terminal region contributes to promoting RNase P catalysis in ways other than binding to M1 RNA. © 2000 Academic Press

Key Words: **RNase P; C5 protein; deletion derivatives; MBP-fusion.**

Ribonuclease P (RNase P) of *Escherichia coli* is an endonuclease that consists of two subunits (1), an RNA of 377 nucleotides (M1 RNA) and a highly basic protein of 119 residues (C5 protein). This enzyme processes the 5′ leader sequences of precursor tRNAs (ptRNAs) to generate the mature 5′ ends. In the absence of C5 protein, M1 RNA alone is catalytically active under the condition of elevated mono- and divalent cationic concentrations *in vitro* (2). However, both M1 RNA and C5 protein are essential for the RNase P function *in vivo* (3, 4) and for the efficient *in vitro* reaction under physiological conditions (2).

C5 protein has been known to aid the catalytic reaction of M1 RNA by stabilizing the catalytically active conformation of M1 RNA (5) and modulating substrate specificity (6–8). The tertiary structure of the RNase P protein of *Bacillus subtilis* has been determined by an X-ray crystallographic analysis (9). It adopts the fold of an α–β sandwich with a globular structure, and it contains three likely RNA-binding regions which may interact with the ribozyme and substrates. The structure of C5 protein is thought to be basically similar to the RNase P protein of *B. subtilis* because the RNase P protein of *B. subtilis* can functionally replace C5 protein (2). Furthermore, between them, there is a core of highly conserved basic and hydrophobic residues located in the central region, as well as a number of conserved hydrophobic residues in the N-terminal and C-terminal regions (10). The central conserved region is called the RNR motif because it contains the distinct Arg-Asn-Arg sequence (9).

The functional role of C5 protein to promote RNase P catalysis was studied by generating its point mutant derivatives in the conserved hydrophobic and basic residues *in vivo* and *in vitro* (11). Although this study revealed some amino acids that affect its function or the substrate specificity of the RNase P holoenzyme, it was not clear whether the mutation effects resulted from the RNA binding-defect or from other functional defects. Here, we constructed deletion derivatives of C5 protein as MBP-fusion proteins and assessed the deletion effects on promoting the RNase P catalysis and on binding to M1 RNA.

MATERIALS AND METHODS

Plasmid construction. To construct plasmids expressing MBP-C5 fusion protein or its deletion derivatives, the corresponding DNA fragments of the *rnp*A gene encoding C5 protein were amplified from a semisynthetic *rnp*A gene of pKKRNPd23 by PCR (12). The amplified DNA fragments were digested with *Bam*HI and *Pst*I, and cloned into pMAL-c2 (New England Biolabs), the MBP-fusion vector. The primers used are shown in Table 1. Primer N0 was paired with C0 to generate the intact MBP-C5 fusion protein (MC5). Primer N0 was also paired with C19, C39, C47, and C59 to generate plasmids pMC5ΔC19, pMC5ΔC39, pMC5ΔC47, and pMC5ΔC59, respectively, which expressed the C-terminal deletion proteins. Likewise, primer C0 was also paired with N13, N21, N39, N45, and N65 to generate plasmids pMC5ΔN13, pMC5ΔN21, pMC5ΔN39, pMC5ΔN45, and pMC5ΔN65, respectively, which expressed the N-terminal deletion

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TABLE 1

PCR Primers Used to Construct Plasmids Expressing MC5 Protein or Its Derivatives

Primer	Sequence of primer
N0	5′-CGCGGATCCATGGTTAAACTGGCATTC-3′
N13	5′-CGCGGATCCACTCCCAGTCAATTCACA-3′
N21	5′-CGCGGATCCTTCCAGCAGCCACAACGG-3′
N39	5′-CGCGGATCCAATTCGCTGGGGCATCCC-3′
N45	5′-CGCGGATCCCGTAGCGGTCTTACAGTC-3′
N65	5′-CGCGGATCCAAACGTCTGACGCGTGAA-3′
C0	5′-AAACTGCAGTCAGGACCCGCGAGCCAG-3′
C19	5′-AAACTGCAGTCAAGCACGGTTATCGAGGTC-3′
C39	5′-AAACTGCAGTCAGAGTTCATGTTGGCGCAG-3′
C47	5′-AAACTGCAGTCAGCGTTCATGGGCGCGTCG-3′
C59	5′-AAACTGCAGTCAATGGGCGCGTCGAACGTT-3′

proteins. Primers N39 and C47 were used to generate pMC5ΔN39ΔC47. All PCR products were verified by DNA sequencing.

Overexpression and purification of MBP-fusion proteins. *E. coli* JM109 (13) cells containing plasmids were grown to an A_{600} of 0.6, and then induced with 0.3 mM IPTG for 2 h. The cell extract was prepared by sonication after adding lysozyme and the protease inhibitor cocktail (Pharmacia) in column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 10 mM β-mercapthoethanol). It was subsequently centrifuged at 25,000*g* for 30 min at 4°C. The supernatant was loaded onto an amylose affinity column, and the MBP-fusion protein was eluted with 10 mM maltose according to the manufacturer's instruction (New England Biolabs). The final protein fraction was dialyzed in the RNase P reaction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NH_4Cl, 10 mM $MgCl_2$). The MBP protein expressed from the pMAL-c2 vector was also purified as described above and used as a control protein.

Assay for RNase P activity. M1 RNA was synthesized by *in vitro* run-off transcription with SP6 RNA polymerase using *Fok*I-cleaved pGER3 DNA as a template (12). Precursor $tRNA^{Phe}$ was generated by *in vitro* transcription with T7 RNA polymerase using *Bst*N1-cleaved pBO201 (Park, B. H. unpublished results) and internally labeled with [α-^{32}P]CTP. The RNase P reaction of 10–100 nM $ptRNA^{Phe}$ was carried out with 1–10 nM M1 RNA in the absence of and presence of MBP-fusion proteins in the RNase P reaction buffer. The reaction mixture was incubated for 30 min at 37°C, and it was quenched by extracting with phenol:chloroform:isoamyl alcohol (25:24:1). The RNA was ethanol-precipitated and analyzed by electrophoresis on a 5% polyacrylamide gel containing 7 M urea.

Gel mobility-shift assay. The internally ^{32}P-labeled M1 RNA of 0.1–10 nM was dissolved in 18 μl of RNA-binding buffer [20 mM K-Hepes, pH 8.0, 400 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 0.01% (v/v) Nonidet P-40, 5% (v/v) glycerol]. Proteins were serially diluted to the desired concentrations in the same buffer. The binding assay was initiated by adding 2 μl of the protein to the RNA solution to the final volume of 20 μl. After a 5-min incubation at 37°C, 10 μl of the reaction mixture was loaded onto a 5% polyacrylamide gel (acrylamide:N,N′-methylene-bis acrylamide, 79:1). The gel was run at a constant current of 13 mA for 2 h with recirculation of the 0.25 × TBE. After electrophoresis, the gel was dried and analyzed with a Molecular Dynamics PhophorImager.

Determination of the M1 RNA contents of the purified protein fractions. Each purified protein of 20 pmol was extracted with phenol:chroloform:isoamyl alcohol (25:24:1). The extracted RNA was electrophresed on a 5% polyacrylamide gel containing 7 M urea and electrotransferred onto a Hybond-N^+ membrane (Amersham). Hybridization was performed according to the manufacturer's instruction with the anti-sense M1 RNA probe, which was prepared as described (14). Amounts of M1 RNA were estimated by analyzing the filter with a Molecular Dynamics PhosphorImager.

Complementation assay. *E. coli* A49 (3) cells containing the *rnp*A49 mutation was used for a complementation assay. Complementation tests were performed by examining the colony formation of *E. coli* A49 cells containing plasmids on LB plates supplemented with 50 μg/ml ampicillin in the absence or presence of 0.1 mM IPTG.

RESULTS

Purification of MBP-C5 Fusion Protein and Its Derivatives

The purification of functional C5 protein in high concentrations is almost impossible due to its low solubility. In this work, we expressed the recombinant C5 protein as a MBP-fusion protein (Fig. 1) because we thought that the presence of the soluble MBP moiety could make the fusion protein more soluble. Upon IPTG induction *E. coli* strain JM109 containing pMC5 accumulated the MBP-C5 fusion protein (MC5). MC5 was purified as a soluble protein under native conditions by amylose affinity column chromatography. SDS-polyacrylamide gel electrophoresis and Coomassie blue staining of the purified recombinant protein revealed a single band of the expected size with a very minor band of endogenous MBP (data not shown). The recombinant protein allowed M1 RNA to complete 5′-end processing of an *E. coli* $ptRNA^{Phe}$ in the 10 mM Mg^{2+}-containing buffer (Fig. 2A). The product formation in the excess substrate condition was saturated when the molar ratio between MC5 and M1 RNA was more than 1:1 (data not shown), suggesting that most of the recombinant protein was functional. The constructs expressing deletion derivatives of C5 protein fused to MBP were prepared by cloning the corresponding coding sequences into the MBP fusion vector (Fig. 1). All of the deletion derivatives, except MC5ΔN39ΔC47, were purified by amylose affinity chromatography with the same method that was used for purification of the MC5 protein. Although we observed that MC5ΔN39ΔC47 was overexpressed in the cell, we were unable to purify it because it was rapidly degraded during the purification even in the presence of protease inhibitors. MC5ΔN13, MC5ΔN21, MC5ΔC19 MC5ΔN39, MC5ΔC19, MC5ΔC39, MC5ΔN47, and MC5ΔC59 were purified to near homogeneity like MC5. However, MC5ΔN39, MC5ΔN45, and MC5ΔN65 were contaminated with a fast-migrating protein band which might be the degraded product at the C-terminus during the purification. The fast-migrating protein constitutes about 30% of the total purified protein. The concentrations of these proteins were calculated by deducting the amount of the degraded product from the amount of the total purified protein on the basis of the band intensities on the SDS-polyacrylamide gel.

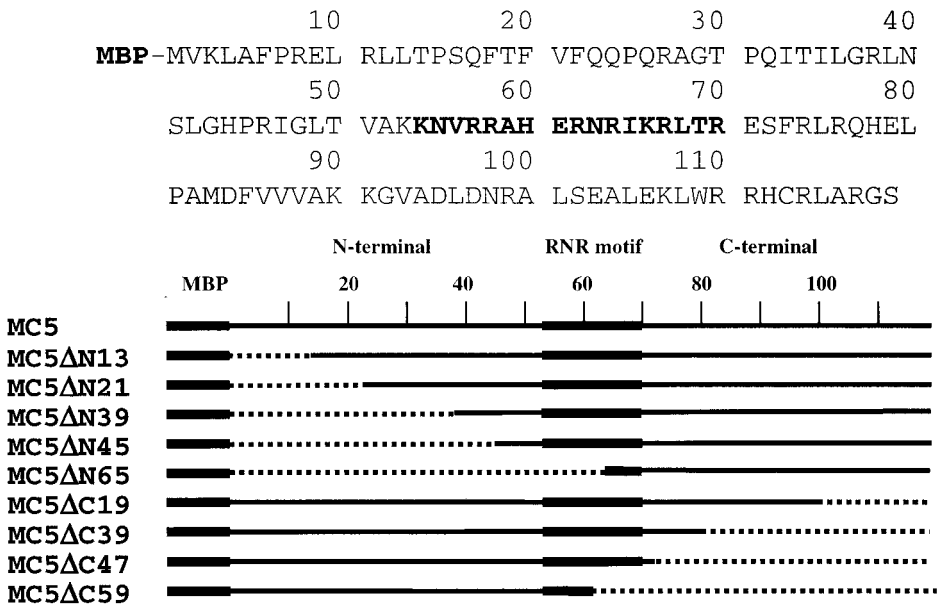

FIG. 1. Construction of the deletion derivatives of C5 protein in MBP-fusions. The 119 amino acid residues of C5 protein are listed after the MBP moiety. The dotted line of the deletion derivative stands for the deleted region. The numbers of the proteins indicate the deleted residues from the N-terminus or C-terminus. The characters in bold correspond to the RNR motif, which is conserved among bacterial RNase P proteins.

Effects of the Deletions in C5 Protein on RNase P Catalysis

The ability of the deletion derivatives of MC5 protein to enhance the cleavage reaction of $ptRNA^{Phe}$ catalyzed by M1 RNA was examined in the 10 mM Mg^{2+} buffer, where the rate of M1 RNA alone was not sufficient to achieve processing. Most of the deletion derivatives showed RNase P activity in the presence of an excess (20-fold) of the protein to M1 RNA (Fig. 2). The percent cleavage of $ptRNA^{Phe}$ by the deletion derivatives was calculated with the amounts of reaction products catalyzed only by the *in vitro* assembled holoenzyme (Table 2). These amounts were obtained by subtracting the amounts of reaction products catalyzed by the protein alone, from the total amounts of the reaction products. Derivatives missing up to 21 residues at the N-terminus or 39 residues at the C-terminus were able to efficiently promote RNase P activity at high protein concentrations. Further deletions significantly decreased the promoting activity. The deletion from the N-terminus into some residues in the central conserved region, called the RNR motif, as in MC5ΔN65, particularly caused a nearly complete loss of the activity.

In Vitro M1 RNA-Binding Ability of the Deletion Derivatives

To see whether the decrease in the activity of the deletion derivatives resulted from RNA-binding defects, complex formation of the proteins with M1 RNA (Fig. 3) was examined and equilibrium dissociation constants (K_d) of the protein-M1 RNA complexes (Table 3) were determined by conducting gel mobility-shift assays. Only some N-terminal deletion derivatives, MC5ΔN13, MC5ΔN21, MC5ΔN39, and MC5ΔN45 showed binding affinity (K_d = 30–140 nM) to M1 RNA, although their interactions were much weaker than the interaction of MC5 protein (K_d = 0.4 nM). The derivatives with the deletions in the C-terminal region or the RNR motif did not show any specific affinity to M1 RNA. Therefore, the C-terminal region, as well as the RNR motif, seems to be essential for the binding of C5 protein to M1 RNA.

M1 RNA Contents in the Purified Proteins

The deletion derivatives showed some cleavage activity of $ptRNA^{Phe}$ in the absence of M1 RNA added in the reaction mixture. This finding suggests that a proportion of the deletion derivative was purified as the holoenzyme complex. The proportion of the holoenzyme in the purified deletion derivative was determined from the amounts of M1 RNA contained in the protein fraction by Northern analysis (Fig. 4 and Table 3). The proportions of the holoenzyme in the purified MC5, MC5ΔN39, and MC5ΔN45 were 0.54%, 0.07%, and 0.05% in the mole ratio, respectively. While MC5ΔN13, MC5ΔN21, MC5ΔC19, MC5ΔC39, and MC5ΔC47 exhibited the proportions of less than 0.02%, MC5ΔN65 and MC5ΔC59 showed no M1 RNA contents in the protein preparations. Since RNase P activity displayed by the purified proteins alone correlated to

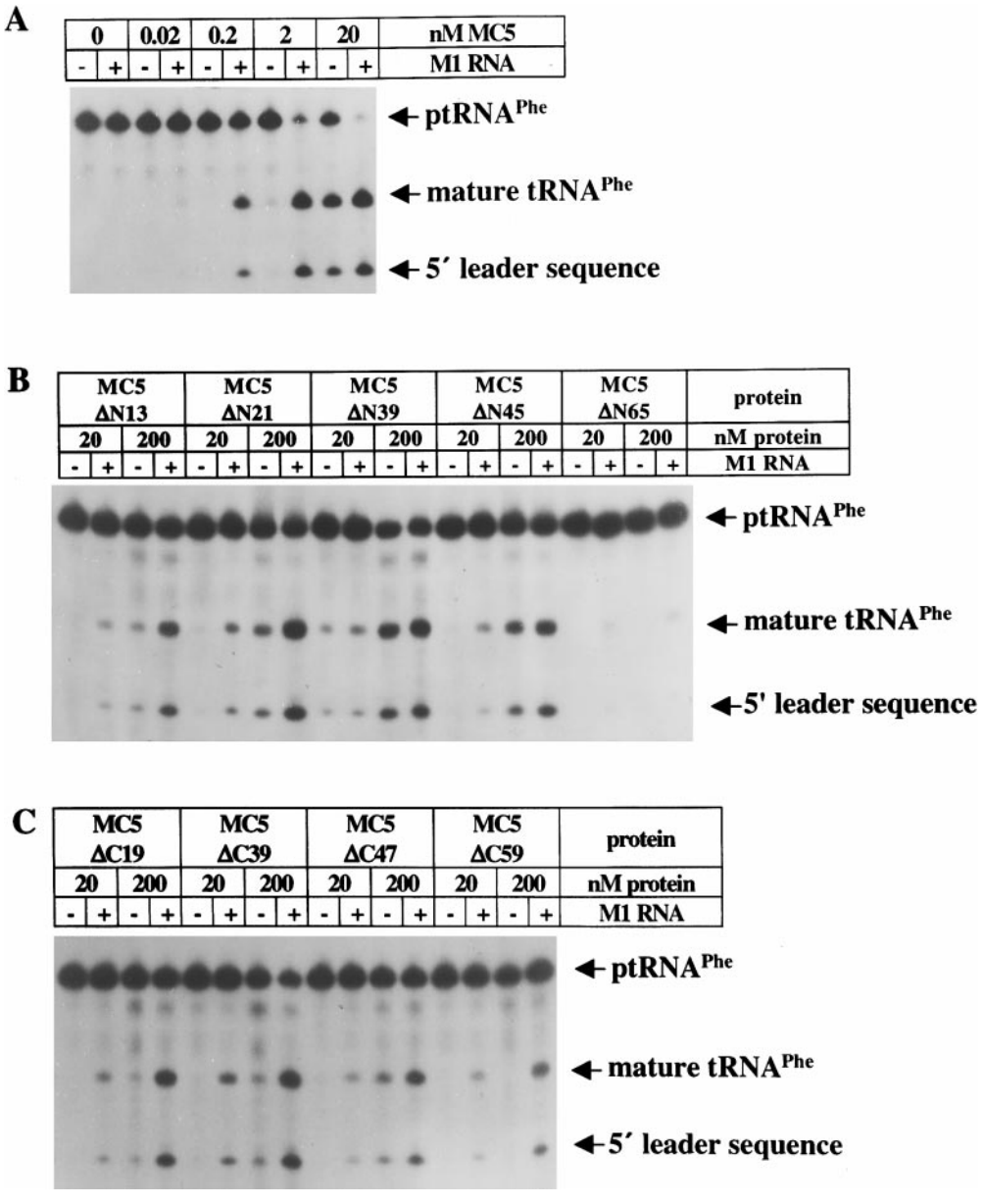

FIG. 2. Effects of the deletion in C5 protein on the RNase P reaction with ptRNA^{Phe} as a substrate. (A) The RNase P reaction with MC5; (B) with the N-terminal deletion derivatives; (C) with the C-terminal deletion derivatives. The 10 nM ptRNA^{Phe} was processed with indicated concentrations of MC5, or its deletion derivatives in the absence or presence of 10 nM M1 RNA at 37°C for 30 min. The cleavage products were analyzed in 5% polyacrylamide gels containing 7 M urea.

the amounts of M1 RNA carried by the proteins themselves (Fig. 2), it seems that the deletion derivatives of MC5 could form the holoenyzme complexes with M1 RNA *in vivo*. However, we do not know whether the M1 RNA contents refer to the ability of the protein to interact with M1 RNA *in vivo*, because MC5ΔN13 and MC5ΔN21, the M1 RNA contents of which were comparable to those of MC5ΔC19, MC5ΔC39, and MC5ΔC47, were able to effectively bind to M1 RNA *in vitro*, while MC5ΔC19, MC5ΔC39, and MC5ΔC47 were not. This discrepancy may be due to different efficiencies in the ribonucleoprotein assembly between *in vivo* and *in vitro*. Alternatively, it may be explained simply by differential stabilities of the *in vivo* assembled holoenzymes.

Function of Deletion Derivatives in Vivo

The plasmids expressing MBP fusion proteins were introduced into a temperature-sensitive mutant *E. coli* strain A49 carrying the *rnpA49* mutation that is responsible for the thermosensitivity of C5 protein function (15). The transformed cells were tested for their ability to complement the growth defect of the cells at

TABLE 2

Cleavage Reaction of ptRNA^{Phe} and Complementation of the ts Phenotype of A49 Cells Using MC5 or Its Deletion Derivatives

Protein	% Cleavage[a]	Complementation[b] −IPTG	Complementation[b] +IPTG
No protein	3.2 ± 0.5	−	−
MBP	3.2 ± 0.6	−	−
MC5	34.6 ± 5.2	++	++
MC5ΔN13	17.6 ± 2.5	++	++
MC5ΔN21	37.1 ± 4.3	++	++
MC5ΔN39	9.3 ± 2.5	+	+
MC5ΔN45	13.9 ± 3.0	+	+
MC5ΔN65	3.2 ± 0.6	−	+
MC5ΔC19	22.7 ± 5.7	−	+
MC5ΔC39	43.9 ± 4.9	−	+
MC5ΔC47	6.7 ± 1.4	−	+
MC5ΔC59	10.0 ± 2.5	−	+
MC5ΔN39ΔC47	ND	−	−

[a] The percentage cleavage of ptRNA^{Phe} was measured in the RNase P reaction with 10 nM M1 RNA and 200 nM deletion derivatives or MBP. In the case of MC5, 0.2 nM protein was used due to its high RNase P promoting activity in the protein excess condition. Nearly 100% cleavage was observed in the presence of 2 nM MC5. All quantitations are means of at least three different determinations. ND, not determined.

[b] Complementation assay was performed by observing the colony formation by *E. coli* A49 cells containing plasmids on LB/ampicillin medium plate at 42°C, in the presence or absence of 0.1 mM IPTG. The derivatives were classified into three categories, depending on colony sizes after the plates were incubated for 18 h. If the colony size observed with the plasmid expressing the deletion derivative was comparable to that of plasmid pMC5, the deletion derivative was classified as ++ (the colony diameter was about 2 mm); the deletion derivative which showed no visible colony formation, like the untransformed A49 cells, was classified as −. The intermediate classification, indicated as + (the colony diameter was 0.3–1 mm), refers to weak or moderate complementation of the ts phenotype of A49 cells.

the nonpermissive temperature (Table 2). Expression of the fusion proteins was under the control of an IPTG-inducible *tac* promoter, which allows a low level of transcription even in the uninduced state. Since the absence and presence of IPTG could represent a low and high level expression of the fusion proteins, respectively, the complementation analysis was performed in the absence and presence of IPTG. In the absence of IPTG, the N-terminal deletion plasmids pMC5ΔN13 and pMC5ΔN21 fully complemented the ts phenotype of A49 cells as the wild type plasmid pMC5 did. Plasmids pMC5ΔN39 and pMC5ΔN45 exhibited moderate complementing effects. In contrast, plasmids expressing the C-terminal or RNR motif deletion derivatives, pMC5ΔN65, pMC5ΔC19, pMC5ΔC39, pMC5ΔC47, and pMC5ΔN59, failed to exhibit their ability to complement the ts phenotype of A49 cells. Their overexpression with 0.1 mM IPTG, however, showed moderate complementation. Plasmid pMC5ΔN39ΔC47 did not demonstrate any complementation ability, even in the presence of 0.1 mM IPTG.

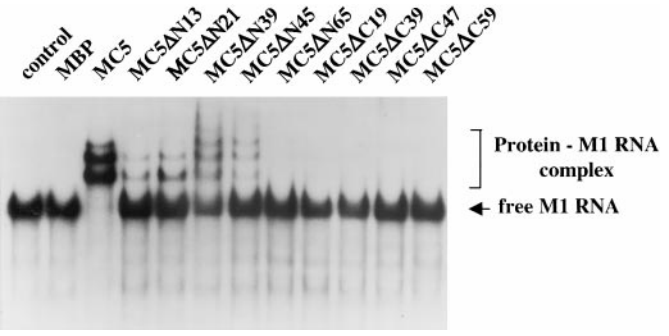

FIG. 3. Complex formation of MC5 protein or its deletion derivatives with M1 RNA. The mixture of 100 nM protein and 10 nM M1 RNA was incubated in the binding buffer at 37°C for 5 min. Complex formation between the protein and M1 RNA was analyzed by a gel mobility-shift assay. In the control experiment, no protein was added.

DISCUSSION

In this study, we first described a procedure to purify soluble C5 protein as a MBP-fusion in high yield, under native conditions, by a single-step procedure, and demonstrated that the fusion protein MC5 was functional *in vitro* and *in vivo*. We also generated derivatives of MC5 having deletions in the C5 protein residues. Then, we tested the RNase P-promoting activity of the deletion derivatives in order to determine which parts of the protein molecule are important for C5 protein functions. Surprisingly, derivatives with the N-terminal or the C-terminal deletions nearly up to half of the total

TABLE 3

The Binding Affinity to M1 RNA *in Vitro* and the M1 RNA Contents of Purified Deletion Derivatives of MC5 Protein

Protein	Dissociation constant[a] K_d (nM)	M1 RNA contents[b] (fmol)
MBP	—[c]	0.0
MC5	0.4	107.2
MC5ΔN13	140	0.8
MC5ΔN21	100	1.5
MC5ΔN39	30	14.6
MC5ΔN45	100	9.8
MC5ΔN65	—	0.0
MC5ΔC19	—	1.8
MC5ΔC39	—	1.7
MC5ΔC47	—	3.4
MC5ΔC59	—	0.0

[a] Dissociation constants were calculated on the basis of gel mobility-shift assays.

[b] M1 RNA contents in 20 pmol of the purified proteins.

[c] No binding affinity was observed under the experimental conditions.

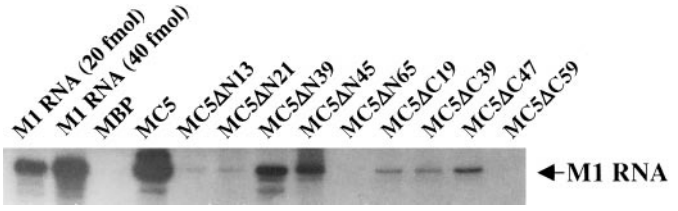

FIG. 4. M1 RNA contents in the purified MC5 protein or its deletion derivatives. RNA extracted from the purified protein was fractionated in a 5% polyacrylamide gel containing 7 M urea and subjected to Northern analysis with the antisense M1 RNA probe. Each band represents the M1 RNA content in 20 pmol of the purified deletion protein.

residues showed a significant RNase P-promoting activity under the protein excess condition. This finding indicated that the intact sequence of C5 protein is not required for the promoting activity.

The N-terminal and the C-terminal deletions showed different effects on RNase P functions. While the N-terminal deletion derivatives retained binding ability to M1 RNA, the C-terminal deletion derivatives showed no detectable binding *in vitro.* Therefore, the C-terminal region appears to be essential for the binding of C5 protein to M1 RNA. We also observed that the N-terminal deletion derivatives were able to at least moderately complement the ts phenotype of *E. coli* A49 cells with basal-level expression, while the C-terminal deletion derivatives were not if they were not overexpressed. This *in vivo* results could be explained by the difference between the binding abilities of the N-terminal and C-terminal deletion derivatives. The RNR motif also seems to be involved in binding to M1 RNA, because the extension of the N-terminal deletion to the RNR motif, as in pMC5ΔN65, resulted in the loss of the M1 RNA binding ability. The RNR motif and C-terminal region may correspond to the region extended from helix B, through β-strand 4, to helix C in the tertiary structure of RNase P protein of *B. subtilis.* This region has been reported as a candidate for an RNA-binding domain (9).

In spite of lack of M1 RNA binding ability of the C-terminal deletion derivatives, their efficiencies in promoting RNase P catalysis were comparable to that of the N-terminal deletion derivatives. Therefore, the N-terminal region should contribute to promoting RNase P catalysis in ways other than binding to M1 RNA. In this respect, it was noteworthy that the N-terminal region of C5 protein corresponds to the residues constituting most of the central craft in the tertiary structure of RNase P protein of *B. subtilis* (9), which was suggested to interact with the 5′ leader sequence of ptRNA in the holoenzyme-substrate complex (16, 17).

There are some discrepancies in correlating the *in vitro* activities of the deletion derivatives with the abilities of complementing the ts phenotype of A49 cells. Although MC5ΔN13 and MC5ΔN21 catalyzes the cleavage of $ptRNA^{Phe}$ quite efficiently only at high concentrations, they are able to support full complementation *in vivo.* In contrast, MC5ΔC19 and MC5ΔC39, which show *in vitro* activities comparable to MC5ΔN13 and MC5ΔN21, exhibit moderate complementation only when their expression is induced with IPTG. These discrepancies may not be simply explained because RNase P acts on various substrates *in vivo* (18–20), and C5 protein affects RNase P catalysis in a substrate identity-dependent manner (6–8). However, both *in vivo* and *in vitro* results provide useful inferences that can be used to assess the importance of specific regions in C5 protein to its functions.

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