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The Carboxyl-terminal Extension of Yeast tRNA m^5C Methyltransferase Enhances the Catalytic Efficiency of the Amino-terminal Domain

The human tRNA m^5C methyltransferase is a potential target for anticancer drugs because it is a novel downstream target of the proto-oncogene myc, mediating Myc-induced cell proliferation. Sequence comparisons of RNA m^5C methyltransferases indicate that the eukaryotic enzymes possess, in addition to a conserved catalytic domain, a large characteristic carboxyl-terminal extension. To gain insight into the function of this additional domain, the modular architecture of the yeast tRNA m^5C methyltransferase orthologue, Trm4p, was studied. The yeast enzyme catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to carbon 5 of cytosine at different positions depending on the tRNAs. By limited proteolysis, Trm4p was shown to be composed of two domains that have been separately produced and purified. Here we demonstrate that the amino-terminal domain, encompassing the active site, binds tRNA with similar affinity as the whole enzyme but shows low catalytic efficiency. The carboxyl-terminal domain displays only weak affinity for tRNA. It is not required for m^5C formation and does not appear to contribute to substrate specificity. However, it enhances considerably the catalytic efficiency of the amino-terminal domain.

Maturation of RNAs is a complex process that involves numerous post-transcriptional modification enzymes. Transfer RNAs present the largest variety of chemical modifications and the highest degree of modification among all RNA species. Modifications at position 34 of the anticodon or 37, 3' to the anticodon, are important for fidelity during decoding of genetic information. The function of most of the modifications outside the anticodon loop remains to be characterized. Yet some of these modifications have been shown to play a role in the stabilization of the tRNA tertiary structure, the correct folding of tRNA, and the specific recognition of tRNAs by the cognate aminoacyl-tRNA synthetases or translation factors (1–4).

The methylations at different base positions and at the 2'-hydroxyl group of ribose are the most frequently encountered modifications in all RNAs. Among them, 5-methylcytosine (m^5C) is found in tRNAs of eukaryotic and archaeal organisms but not in eubacteria. By increasing the hydrophobicity of the nucleoside and thus its propensity for base stacking, methylation of cytosine at C5 helps to stabilize the nucleic acid structure (1, 5). In Saccharomyces cerevisiae, its formation is catalyzed by a multisite-specific tRNA m^5C methyltransferase (MTase), Trm4p, that uses S-adenosyl-L-methionine (AdoMet) as methyl donor. Depending on the tRNA substrate, the target cytosines are located at positions 34, 40, 48, and/or 49 (6). Most tRNAs are modified at positions 48 and/or 49, whereas methylations at positions 34 and 40, present only in tRNA^{Leu} and tRNA^{Phe}, respectively, occur exclusively in intron-containing tRNA precursors.

The sequence and structure comparisons of different RNA m^5C MTases (7–9) indicate that the amino-terminal region of Trm4p, which bears the AdoMet-binding sequence motifs and the catalytic residues, including the two crucial cysteines (10, 11), adopts the Rossmann-like fold present in the vast majority of AdoMet-dependent MTases (12, 13) (Fig. 1). A major characteristic of the eukaryotic RNA m^5C MTases is the presence of a polypeptide chain extension appended to the carboxyl terminus of the protein (7) (Fig. 1). The carboxyl-terminal extension of Trm4p is unrelated to known RNA-binding domains or any other domain with known function according to a BLAST similarity search. The human orthologue of Trm4p was identified as the best match of a BLAST search in the human UniGene database using Trm4p as a query, and its cDNA was expressed in a yeast strain deprived of the endogenous TRM4 gene (14). This human tRNA m^5C MTase was shown to exhibit a much narrower specificity than Trm4p, because it catalyzes only the intron-dependent formation of m^5C34 and not those of m^5C48 and m^5C49 in human or yeast tRNA substrates. This enzyme, also called Misu or NSun2, is a novel downstream target of the proto-oncogene myc, mediating the effects of Myc on cell growth and proliferation (15). It is overexpressed in different types of tumors and is a potential target for cancer therapies because the repression of its mRNA expression reduces tumor growth.
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| Subfamilies | Representative members | Additional domain | Core domain | Additional domain |
|-------------|------------------------|-------------------|-------------|------------------|
| I           | E. coli 16s tRNA mC967 MTase | NTI               | X*-4II-III沃 V-Me*III | NTI |
| II          | E. coli 16s tRNA mC1407 MTase | NTI               | X*-4II-III沃 V-Me*III | NTI |
| V           | P. hortensis PH1374 | NTI               | X*-4II-III沃 V-Me*III | NTI |
| VII         | S. cerevisiae tRNA mC MTase | NTI               | X*-4II-III沃 V-Me*III | NTI |

FIGURE 1. Schematic arrangement of the conserved regions in 4 of the 12 subfamilies of RNA mC MTases (7, 8). The core domain is constituted of a small region and a larger catalytic MTase region (in black), including the eight conserved characteristic motifs.

formation in vivo. The comparison of the human and yeast enzymes, which display close sequence similarities (supplemental Fig. S1) but target different cytosines in tRNAs, should help to better understand the relationship between amino acid sequence and substrate specificity.

Although sequence analysis indicates the modular arrangement of the majority of RNA-modifying enzymes and identifies potential RNA-binding domains (16–18), individual domains have been produced and characterized only in a few cases (19, 20). In this work, we have probed the modular architecture of yeast tRNA mC MTase (Trm4p) by limited proteolysis. The coding sequences of the two identified structural domains were then cloned and expressed in *Escherichia coli* to examine the role of the purified corresponding proteins in tRNA binding and catalytic activity and gain insight into the function of the carboxyl-terminal domain.

EXPERIMENTAL PROCEDURES

Cloning of Trm4p Domains Coding Sequences—Recombinant versions of NTrm4p and CTrm4p were constructed with a His$_6$ tag at their amino- and carboxyl-terminal ends, respectively. The open reading frames were amplified from yeast genomic DNA using the PCR primers AAAACATGGGTCA-TCATCATCATCATCAGGTCAGAAGAAGAAGTTTCAAAAAAAG (5’ primer) and AAAAGATCTCATTGTGTT-TGGAGGTCTCCAGAC (3’ primer) for NTrm4p and AAAACATGGGTAAATATGGAGTTAGAAGAAGTCAAATAG (5’ primer) and AAAAGATCTCATTGTGTT-TGGAGGTCTCCAGAC (3’ primer) for CTrm4p. The 5’ primers created the ATG start codon in the Ncol restriction site, and the 3’ primers introduce a BamHI restriction site immediately downstream from the TGA stop codon. The six histidine codons were introduced by the 5’ primer after the ATG start codon followed by a glycine codon for NTrm4p construct and by the 3’ primer immediately upstream from the TGA stop codon for CTrm4p. The amplified fragments were cut with Ncol and BamHI restriction enzymes and were cloned into the same sites in the expression vector pET11d (Novagen).

Protein Overexpression and Purification—Trm4p was expressed and purified as described previously (11). NTrm4p and CTrm4p encoded by the recombinant plasmids were expressed in the *E. coli* BL21(DE3)~host strain (Novagen). The bacteria were grown in MM9 minimal medium supplemented with 1 mM MgSO$_4$, 0.3 mM CaCl$_2$, 0.2% glucose, 0.5% casamino acids, 30 µg/ml chloramphenicol, and 100 µg/ml ampicillin. Cultures (1 liter) were grown at 37 °C to an A$_{600}$ of 0.8 and transferred at 20 °C, and expression was induced by addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1 mM. After 3 h of induction, the cells were collected by centrifugation. All purification steps were conducted at 4 °C. *E. coli* cells expressing the recombinant enzymes were resuspended in 5 volumes of buffer A (0.3 mM NaCl, 10 mM β-mercaptoethanol, 1% (v/v) protease inhibitor mixture for use in purification of histidine-tagged proteins (Sigma), 50 mM sodium phosphate pH 8) supplemented with 20 mM imidazole. Cells were disrupted by sonication, and the lysate was centrifuged at 30,000 × g for 30 min. The supernatant was loaded on Ni$^{2+}$-nitrilotriacetic acid affinity column (Qiagen) equilibrated in buffer A supplemented with 30 mM imidazole and eluted with buffer A supplemented with 150 mM imidazole. EDTA at a final concentration of 5 mM was added to the eluted protein fractions that were then dialyzed overnight at 4 °C against 20 mM Tris-HCl, pH 8, 0.2 M NaCl, 2 mM dithiothreitol, 5 mM EDTA, 50% glycerol and stored at −80 °C.

Trypsin Limited Proteolysis—Purified recombinant Trm4p (0.13 mg) was incubated in the presence of 0.1% (w/w) dipher-nycarbamyl chloride-treated trypsin (Sigma) in 0.13 M Tris-Cl, pH 8, 0.2 M NaCl, 2 mM dithiothreitol. After 30 min of incubation at 30 °C, the reaction was stopped by addition of the irreversible serine protease inhibitor 4-(2-aminoethyl)-benzenesulfonyl fluoride at 4 mM final concentration (Pefabloc SC, Euromedex), and the sample was immediately analyzed using 12% SDS-polycrylamide gel.

Amino-terminal Sequencing and Mass Spectrometry—Edn degradation of the polypeptide fragments resulting from limited proteolysis was performed by Diep Lê (LEBS, CNRS) using an ABI 407A automated sequencer. The samples were eluted from an SDS-polycrylamide gel on a polyvinylidene difluoride membrane (GE Healthcare) by semidry electrophoresis, and five sequencing cycles were performed. Mass spectrometry was performed on an Applied Biosystems Voyager System 4080 by Christophe Marchand (Institut de Biochimie et Biophysique Moléculaire et Cellulaire, Paris XI). The samples were dialyzed against 10 mM phosphate buffer, pH 8, and mixed with sinapinic acid as a matrix.

Preparation of RNA Substrates—Nonradiolabeled and [α-32P]CTP tRNA transcripts were obtained by *in vitro* transcription with T7 RNA polymerase of linearized plasmids and purification of the resulting transcripts on urea gels as described (21). Poly(C) RNA was bought from Roche Applied Science. Total yeast tRNA from the ΔYBL024w-disrupted yeast strain (lacking Trm4p activity) (6) was prepared as described (22).

Measurement of RNA Binding Affinity—The nitrocellulose-binding assay uses the preferential retention by nitrocellulose of proteins and protein-nucleic acid complexes but not of free nucleic acids. The amount of radiolabeled tRNA complexed with protein in a solution is quantified by filtering through a nitrocellulose membrane and measuring the amount of retained radioactivity. The affinity of the different proteins for the mature yeast
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Dissociation constants of the various proteins for mature yeast tRNA<sup>Phe</sup> determined by the nitrocellulose-binding assay at 50 mM NaCl

| Protein         | $K_d$ (nM) |
|-----------------|------------|
|                 | -Sinefungin | +Sinefungin |
| Trm4p           | 70 ± 10     | 63 ± 5      |
| NTrm4p          | 30 ± 5      | 25 ± 3      |
| CTrm4p          | 2560 ± 300  | ND<sup>a</sup> |
| NTrm4p + CTrm4p | 29 ± 3      | 39 ± 6      |

<sup>a</sup> ND indicates not determined.

In Vitro tRNA m<sup>5</sup>C MTase Assays—Two different methods were used to determine the m<sup>5</sup>C MTase activity. The first approach used [α-<sup>32</sup>P]CTP-radiolabeled mature yeast tRNA transcripts. 200 nM of enzyme was incubated for 2 h at 30 °C with 60 μM AdoMet (Sigma), 1–2 nM [α-<sup>32</sup>P]CTP-tRNA<sup>Phe</sup>, 1 μM cold tRNA<sup>Phe</sup> in 50 μl of 20 mM Tris-HCl, pH 8, 50 mM NaCl, 2 mM dithiothreitol. The reaction was stopped with 200 μl of phenol:chloroform:isoamyl alcohol (25:24:1), pH 4.5, to precipitate the protein. tRNA in the aqueous phase was extracted by centrifugation at 10,000 × g for 5 min, ethanol-precipitated, and then hydrolyzed to 5'-nucleoside monophosphates by nuclease P1 (ICN) overnight at 37 °C in 50 mM sodium acetate, pH 5.3. Each hydrolysate was analyzed by two-dimensional chromatography on cellulose plates (Macherey-Nagel) as described (21), and the radioactivity in the spots was determined by exposing to a PhosphorImager screen and quantifying by ImageQuant software. The relative amount of m<sup>5</sup>C formed per tRNA molecule was determined by taking into account the total number of cytosines in the tRNA.

The second assay, based on the methyl group incorporation from [<sup>3</sup>H]AdoMet (15 Ci/mmol; GE Healthcare) into nonradioactive RNA transcripts, was carried out in the same buffer and temperature conditions. Kinetics to determine the substrate specificity of NTrm4p compared with that of Trm4p were performed at 50 mM NaCl by incubating 400 nM of enzyme, 2 mM AdoMet and 10 μM total RNA from the ΔYBL024w-disrupted yeast strain for 2 h. The kinetic parameters for the mature yeast tRNA<sup>Phe</sup> transcript were measured by varying the tRNA<sup>Phe</sup> concentration in the range of 0.125–8 μM at 50 mM NaCl or 0.0625–2 μM at 300 mM NaCl. 60 μM AdoMet was used, except in the case of NTrm4p where 80 μM AdoMet was used. The concentrations of Trm4p, NTrm4p:CTrm4p mixture, or NTrm4p were 600 nM, 1 μM, or 1.3 μM at 50 mM NaCl, respectively, and 40 nM for Trm4p or 200 nM for NTrm4p:CTrm4p at 300 mM NaCl. The samples were incubated for 2 or 1 h for Trm4p at 300 mM NaCl. After incubation, all the reactions were stopped by addition of cold 5% trichloroacetic acid, and the precipitates were collected by filtration through GF/C filters (Whatman). The filters were washed with cold 5% trichloroacetic acid and dried, and the radioactivity was measured by liquid scintillation counting.

RESULTS

Yeast tRNA m<sup>5</sup>C MTase Is Composed of Two Domains That Can Be Produced Separately—From the sequence comparison (7–9) and structure determination of different characterized tRNA<sup>Phe</sup> transcript was measured in buffer B (20 mM Tris-HCl, pH 8, 50 mM NaCl) in the presence or absence of 0.1 mM sinefungin (Sigma). After 20 min of incubation at 25 °C, each sample (20 μl) was filtered through a nitrocellulose membrane (type SM113 0.45 μm; Sartorius, Germany) that had been previously washed twice with 500 μl of buffer B at 4 °C. The membranes were then washed twice with 500 μl of buffer B at 4 °C and dried, and the retained radioactivity was measured by liquid scintillation counting.
RNA m5C MTases (9, 23), these enzymes appear to be composed of a catalytic core domain and one or several additional domains (Fig. 1). In particular, yeast tRNA m5C MTase (Trm4p) is an 80-kDa protein that has been previously expressed in *E. coli* as an amino-terminally His-tagged protein (6). To probe its modular organization, the enzyme was subjected to limited trypsin proteolysis. With a 1:1000 trypsin:protein ratio, three main stable polypeptides were obtained after incubation for 30 min at 37 °C, as shown by SDS-PAGE analysis (Fig. 2B). The proteins were extracted from the gel, and their amino-terminal sequence was determined by Edman degradation and their mass by mass spectrometry. The band P1 corresponds to the amino-terminal domain of the protein without the histidine tag (NTrm4p, residues 1–437, 49.3 kDa), whereas the band P2 of similar mass is a truncated version of this domain where the first 22 amino acids are lacking (Fig. 2A). This is in agreement with protein modeling, predicting that these amino-terminal residues are at least partially disordered (8). The band P3 corresponds to the carboxyl-terminal domain (CTrm4p, residues 438–684; 28.1 kDa; see Fig. 2A).

To investigate the individual functional role played by the two domains, we cloned and expressed in *E. coli* the corresponding coding sequences with the addition of a His tag at the amino terminus for the two forms of the amino-terminal domain or at the carboxyl terminus for CTrm4p. The NTrm4p and CTrm4p domains, which were purified by nickel affinity chromatography, showed the expected apparent mass on SDS-PAGE (Fig. 2C). The truncated amino-terminal domain displayed a 4-fold weaker binding affinity for tRNA and had a lower tRNA m5C MTase activity than NTrm4p (data not shown), indicating that the first 22 amino-terminal residues are involved at least in tRNA binding. Therefore, further study of the modular organization of Trm4p was done with the whole NTrm4p.

**NTrm4p Has Similar Affinity for tRNA as the Whole Enzyme whereas CTrm4p Displays Only Weak Affinity for tRNA**—The apparent dissociation constants ($K_d$) for an unmodified mature yeast tRNA$^{\text{Phe}}$ transcript of the different proteins were determined using the nitrocellulose-binding assay (Table 1; Fig. 3). Because AdoMet binding has been shown to induce conforma-
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The Carboxyl-terminal Domain Contributes Considerably to the Efficiency of the Reaction—The role of each domain in the methyl transfer reaction was further analyzed by steady-state kinetics in comparison with full-length enzyme. Because the three active species had different salt-dependent profiles, their kinetic parameters for mature yeast tRNA^Phe were determined both at low (50 mM) and high (300 mM) salt concentrations (Table 2 and Fig. 6), except that the catalytic activity of Trm4p was measurable only at 50 mM NaCl. The catalytic constants for the native enzyme (k_{cat} = 0.015 s^{-1} and K_m = 1.1 mM) at 300 mM NaCl are similar to those reported for E. coli 16 S tRNA m1G967 MTase (26). The Michaelis constants (K_m) for the three active species are similar and do not vary significantly between the two salt concentrations. At 50 mM NaCl, the catalytic efficiency (k_{cat}/K_m) of Trm4p is 5.7-fold lower as compared with Trm4p, resulting from a decrease in k_{cat}. In the presence of an equimolar amount of CTrm4p, the catalytic efficiency of NTrm4p is increased 2-fold, becoming only 3-fold lower than for the whole enzyme. Therefore, the role of the carboxyl-terminal domain is to enhance the catalytic efficiency of the amino-terminal domain mainly by increasing k_{cat}.

This effect is specific of CTrm4p because a 1:1 mixture of NTrm4p

| [NaCl] (mM) | Enzyme | k_{cat} (s^{-1}) | K_m (mM) | k_{cat}/K_m |
|------------|--------|----------------|----------|-------------|
| 50 mM      | Trm4p  | 1.1 ± 0.1      | 1.3 ± 0.1 | 0.85        |
|            | NTrm4p| 0.48 ± 0.04    | 1.7 ± 0.2 | 0.3         |
|            | CTrm4p| 0.19 ± 0.01    | 1.3 ± 0.1 | 0.15        |
| 300 mM     | Trm4p  | 15.0 ± 1.0     | 1.1 ± 0.1 | 13.6        |
|            | NTrm4p| 1.8 ± 0.2      | 0.9 ± 0.1 | 2.0         |
|            | NTrm4p| Not detectable |

The carboxyl-terminal domain of other AdoMet-dependent MTases (24, 25) and because this could influence tRNA binding, the apparent dissociation constants were measured both in the presence or absence of the unreactive AdoMet substrate analogue sinefungin (Table 1). Clearly, the presence of sinefungin has no detectable effect on the K_d values, so we conclude that tRNA binding is probably not affected by a potential AdoMet-dependent conformational change of the enzyme. The apparent K_d value (30 nM) for tRNA^Phe of NTrm4p is of the same order of magnitude as that of the whole enzyme, whereas CTrm4p is capable of binding tRNA^Phe only with a 2.6 μM affinity, indicating that this isolated domain is obviously not an RNA-binding domain (Table 1 and Fig. 3). This is in agreement with the fact that the presence or absence of CTrm4p has apparently no effect on NTrm4p binding to the tRNA substrate.

NTrm4p Is Catalytically Active whereas CTrm4p Is Not—To test the tRNA MTase activity of the different proteins and verify that the product of methyl transfer is m^5C, a mature yeast tRNA^Phe transcript, which is potentially modified solely at position 49, in contrast to its intron-containing precursor which is methylated at both positions 40 and 49 (Fig. 4A—B), was internally labeled with [α-^32P]CTP, subjected to methylation, and hydrolyzed by nuclease P1. The resulting 5’-monophosphate nucleosides were then separated by two-dimensional thin layer chromatography (Fig. 4B). Comparison of migration of the radioactivity to marker nucleosides shows that, after reaction with Trm4p, NTrm4p, or NTrm4p + CTrm4p, the transcript contains m^5C but not in the case of CTrm4p. So CTrm4p alone does not catalyze m^5C formation, as expected, whereas NTrm4p is surprisingly catalytically active per se. Determination of the ratio of radioactivity present in the m^5CMP and CMP spots on the two-dimensional TLC indicates that Trm4p, NTrm4p, and a 1:1 mixture of NTrm4p and CTrm4p catalyze in 2 h the formation of 1.0, 0.4, or 0.7 mol of m^5C per mol of tRNA, respectively. The enhancement of enzymatic activity by addition of CTrm4p to NTrm4p confirms the modular architecture of yeast tRNA m^5C MTase and demonstrates that isolated CTrm4p is important for catalysis by NTrm4p.

Trm4p and NTrm4p Show Different Activity Dependence on Salt Concentration—Before determining the steady-state kinetics parameters of the three active species, Trm4p, NTrm4p, and the 1:1 mixture of NTrm4p and CTrm4p, the dependence on salt concentration of each methyl transfer reaction was examined. Indeed, the enzymatic activity of Trm4p is optimal at high ionic concentration. Therefore, the ability of the different proteins to catalyze transfer of the labeled methyl group of [^3H]AdoMet to unmodified mature yeast tRNA^Phe transcript was tested at increasing NaCl concentrations (Fig. 5). Surprisingly, although the optimum activity of Trm4p and the NTrm4p:CTrm4p mixture was at about 300 mM NaCl, NTrm4p was active only at low salt concentrations. Because the optimum activity of NTrm4p is shifted to that of the whole enzyme in the presence of CTrm4p, this supports the formation of a ternary complex between tRNA, NTrm4p, and CTrm4p during catalysis.

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Y. Motorin, unpublished results.
and bovine serum albumin exhibit the same catalytic constants as NTrm4p alone (data not shown). The role of CTrm4p is best shown at 300 mM NaCl, a salt concentration that is more physiological than 50 mM (27), because in its absence NTrm4p has no detectable activity.

The Amino-terminal Domain Alone Displays Multisite Specificity, Like the Whole Enzyme—To determine whether CTrm4p could play a role in targeting the location of modification in tRNA, the site specificities of Trm4p and NTrm4p were compared by probing the methylation of different RNA transcripts that could be modified only at one of the four possible positions (6) (Fig. 7). In addition, NTrm4p was shown not to have nonspecific activity by using poly(C) RNA as a negative control. Because the different RNA substrates, which are modified in positions 34, 40, 48, or 49 by Trm4p, are also methylated by NTrm4p, both proteins likely possess the same multisite specificity. Therefore, the catalytic core of Trm4p is apparently sufficient to target the four specific sites in the different tRNAs.

DISCUSSION

Sequence analysis (7, 8) and x-ray crystallography (9, 23) of different RNA m⁵C MTases have shown the modular architecture of these enzymes (Fig. 1). A highly conserved structural core domain can be located either at the amino terminus (9) or the carboxyl terminus (23) of an additional structural domain. The core domain is composed of two closely associated regions: a small region and a larger MTase region that bears the catalytic residues and AdoMet-binding motifs. To what extent the additional noncore domains are involved in determining the substrate specificity or whether the core domain alone achieves this task remain important questions to solve. Based on sequence or structural similarities with domains belonging to other RNA-binding proteins (16–18, 28–30), the additional domains were proposed to serve as RNA-binding modules. But this hypothesis has rarely been tested experimentally (see below). This idea is also supported by the existence of two-component RNA-modifying enzymes, such as Gcd10p/Gcd14p (31) and APOBEC1/ACF (32), in which the catalytic activity and RNA specificity depend on different subunits/proteins of the complex.

Here we have examined the modular architecture of yeast tRNA m⁵C MTase, Trm4p, and the function of its two isolated domains. Sequence alignment of different studied RNA m⁵C MTases shows that Trm4p, like the other orthologues from eukaryotes, possesses a large carboxyl-terminal polypeptide extension (7) (Fig. 1), which does not align with any protein or domain of known function in a BLAST search. Trm4p and the orthologous proteins from mammals share 35% sequence identity (supplemental Fig. S1, an E value of 10⁻⁹ in BLAST) with 47% identity for the catalytic core domain and 22% identity for the carboxyl-terminal domain, which indicates a possible similar function for the carboxyl-terminal extensions. Therefore, the yeast enzyme is a potential model for the human enzyme (14) that has been shown to be a novel downstream target of the proto-oncongene myc and thus a potential target for cancer therapies (15). Sequence alignment also indicates that, in several Archaea, the RNA m⁵C MTases contain only the core domain (7) (Fig. 1). This has been confirmed for tRNA m⁵C MTase from Pyrococcus abyssi (33), but it was shown that an archease protein is needed for improving the site-specificity of the MTase. It is therefore interesting to determine whether, in eukaryotic tRNA m⁵C MTases, the amino-terminal domain is sufficient for both catalytic activity and substrate specificity and to investigate the role of the carboxyl-terminal domain.
In this study, both the amino- and carboxyl-terminal domains of yeast tRNA m^5C MTase have been produced separately. The isolated amino-terminal domain is catalytic and the carboxyl-terminal extension is not required for multisite specificity. Yet our results indicate that CTrm4p contributes considerably to the efficiency of the reaction by acting as a trans cofactor for catalysis by NTrm4p. The carboxyl-terminal domain is not an RNA-binding module because it shows only weak affinity for tRNA and does not provide the whole enzyme with RNA-binding properties. This situation contrasts with that described for eukaryotic aminoacyl-tRNA synthetases where the additional noncatalytic domains were shown to be involved in tRNA binding (34–36). Yet our results agree with the few other studies on RNA-modifying enzymes, where one or several domains have been independently produced and characterized. For instance, tRNA m^2,G10 MTase of P. abyssi has been shown by limited trypsin proteolysis to be composed of two domains, a carboxyl-terminal catalytic domain and an amino-terminal THUMP (thiouridine synthases, RNA MTases, and Pseudouridine synthases)-containing domain (19). The THUMP domain, produced as a stand-alone protein, was not able to form a stable complex with tRNA even at a high protein concentration (25 μM), suggesting it is not responsible per se for the affinity of the enzyme for tRNA (K_d = 1 μM). However, it cannot be excluded that the THUMP domain could exhibit its predicted RNA-binding properties (17) only during the steps occurring after substrate recognition. Similarly, biochemical studies of an rRNA m^4A MTase (ErmC'), which mediates anti-

![FIGURE 7. Comparison of the substrate specificities of Trm4p and NTrm4p.](image-url)
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biotic resistance in bacteria and is composed of a catalytic amino-terminal domain and a small carboxyl-terminal domain (37, 38), have shown that the key RNA-binding residues are located in the catalytic amino-terminal domain and that the small additional domain has no important role in RNA binding (39). Nevertheless, a small domain-deleted mutant was completely inactive in vivo and subject to aggregation. Therefore, according to the ErmC’ structure (37, 38), it was suggested that the role of the small domain would be the structural stabilization of the catalytic domain through hydrophobic interactions, especially in the RNA-binding region. Although whole Trm4p and the 1:1 mixture of NTrm4p and CTrm4p were most active at high salt concentrations, NTrm4p was active only at low salt concentrations. In other words, the activity optimum of NTrm4p is shifted from 50 to 300 mM NaCl in the presence of CTrm4p. Therefore, the carboxyl-terminal domain is required for the activity of the catalytic domain at high salt concentrations. Yet CTrm4p does not seem to be as crucial for maintaining the structural integrity of the catalytic domain, as described for ErmC’ (39), because NTrm4p can be produced as an active soluble protein. But it cannot be excluded that CTrm4p could play a role in maintaining an active conformation of NTrm4p, independently of the presence of tRNA, particularly at high salt concentrations.

However, the carboxyl-terminal domain could also have a role in the stabilization of a productive complex between tRNA and the catalytic domain during catalysis through hydrophobic interactions. Indeed, these interactions are stabilized at high salt concentrations, and we observed that the mixture of NTrm4p + CTrm4p is most active at 300 mM NaCl, like the whole enzyme. This catalytically productive ternary complex could result from conformational changes of tRNA, protein, or both occurring in the transition state. For instance, in the case of Pyrococcus furiosus archesoine tRNA guanine transglycosylase, the carboxyl-terminal additional noncatalytic domains do not affect tRNA binding, and it was suggested that their influence would be most significant during the steps following association of the enzyme with its substrate, leading up to and including catalysis (20). This hypothesis is consistent with the tRNA conformational change observed in the crystal structure of the homologous Pyrococcus horikoshii archesoine-tRNA guanine transglycosylase complex in unmodified tRNAVal (28). Indeed, in the new tRNA conformation called the λ form, the carboxyl-terminal extension and PUA (pseudouridine synthases and archesoine-tRNA guanine transglycosylase) domain make many interactions with tRNA, anchoring the acceptor stem. Both enzymes are composed of a catalytic core and three small carboxyl-terminal domains, the last of which, PUA, being identified as a putative RNA-binding domain (16). Several carboxyl-terminal truncated forms of the P. furiosus enzyme have been shown to bind tRNA with similar affinity as the whole enzyme and to be selective for the correct G15 position inside the tRNA molecule, indicating that the carboxyl-terminal domains are not required for activity. However, the PUA domain contributes significantly to the catalytic efficiency because its presence decreases $K_m$ 75-fold and increases $k_{cat}$ 4-fold (20).

For numerous DNA- or RNA-modifying enzymes, the target nucleoside is flipped to gain access to the active site (29, 40). In many cases, the cavity thus generated in the hydrophobic core of the nucleic acid is filled in by protein residues to provide energetic compensation through favorable interactions, mimicking base stacking (41, 42). For instance, in DNA m$^3$C MTase Hhal, Gln-237 from the small additional domain plays a key active role in opening the target C-G base pair before occupying the original position of the flipped cytosine (43). It serves to destabilize the initial binary specific MTase-DNA complex, which in turn lowers the energy barrier for the subsequent base-flipping step. This provides another example of the contribution of a noncatalytic domain to catalysis, through the stabilization of the conformational change of the nucleic acid.

In conclusion, based on all these findings and our results, we propose that the carboxyl-terminal domain of Trm4p may promote the catalytic core domain and/or tRNA substrate to adopt a productive conformation during catalysis, which increases the m$^3$C MTase reaction efficiency. It remains to investigate whether this catalytically productive ternary complex can be linked to a conformational change of tRNA such as base-flipping.

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