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Multisite-specific tRNA:m^{5}C-methyltransferase (Trm4) in yeast *Saccharomyces cerevisiae*: Identification of the gene and substrate specificity of the enzyme

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ABSTRACT

Several genes encoding putative RNA:5-methylcytidine-transferases (m^{5}C-transferases) from different organisms, including yeast, have been identified by sequence homology with the recently identified 16S rRNA:m^{5}C$_{967}$-methyltransferase (gene *SUN*) from *Escherichia coli*. One of the yeast ORFs (YBL024w) was amplified by PCR, inserted in the expression vector pET28b, and the corresponding protein was hyperexpressed in *E. coli* BL21(DE3). The resulting N-terminally His$_6$-tagged recombinant Ybl024p was purified to apparent homogeneity by one-step affinity chromatography on Ni^{2+}-NTA-agarose column. The activity and substrate specificity of the purified Ybl024p were tested in vitro using T7 transcripts of different yeast tRNAs as substrates and S-adenosyl-L-methionine as a donor of the methyl groups. The results indicate that yeast ORF YBL024w encodes S-adenosyl-L-methionine-dependent tRNA: m^{5}C-methyltransferase that is capable of methylating cytosine to m^{5}C at several positions in different yeast tRNAs and pre-tRNAs containing intron. Modification of tRNA occurs at all four positions (34, 40, 48, and 49) at which m^{5}C has been found in yeast tRNAs sequenced so far. Disruption of the ORF YBL024w leads to the complete absence of m^{5}C in total yeast tRNA. Moreover no tRNA:m^{5}C-methyltransferase activity towards all potential m^{5}C methylation sites was detected in the extract of the disrupted yeast strain. These results demonstrate that the protein product of a single gene is responsible for complete m^{5}C methylation of yeast tRNA. Because this newly characterized multisite-specific modification enzyme Ybl024p is the fourth tRNA-specific methyltransferase identified in yeast, we suggest designating it as *TRM4*, the gene corresponding to ORF YBL024w.

Keywords: 5-methylcytidine; anticodon loop; modification; *NCL1*; *NOP2*; one-step gene replacement; *P120*; tRNA; YBL024w; yeast

INTRODUCTION

Among the various posttranscriptionally modified nucleotides present in RNA molecules, those corresponding to methylation at different positions of bases and of the 2′-hydroxyl group of riboses are the most frequently encountered ones. Among these frequent methylated residues, 5-methylcytidine (m^{5}C) is found in various cellular RNAs, but its occurrence depends on the origin and the type of RNA. In all eubacterial organisms, m^{5}C has been found only in ribosomal RNA. For example, two m^{5}C residues were located at positions 967 and 1407 in *Escherichia coli* 16S rRNA and an additional one at position 1962 in 23S rRNA (Noller, 1984; Smith et al., 1992), whereas none of the *E. coli* tRNAs sequenced so far contains m^{5}C (Sprinzl et al., 1998). In contrast, in eukaryotic and archaeal organisms, m^{5}C is found both in tRNAs and in the ribosomal RNA. Indeed, most eukaryotic and archaeal tRNAs are modified to m^{5}C at several different positions, but the most frequently occurring cluster of m^{5}C residues is located at positions 48 and 49 (Sprinzl et al., 1998, reviewed in Auffinger & Westhof, 1998). The positions of at least two m^{5}C residues in *Xenopus laevis* ribosomal 26S RNA have been identified (m^{5}C$_{2944}$ and

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Abbreviations: AdoMet: S-adenosyl-L-methionine; DTT: dithiothreitol; m^{5}C: 5-methylcytidine; ORF: open reading frame; PCR: polymerase chain reaction; tlc: thin-layer chromatography.

$m^5C_{3570/71}$) (Maden, 1988), whereas in other eukaryotic (including yeast) and archaeal rRNAs, the presence of m^5C residues was demonstrated, but their location was not determined (Klootwijk & Planta, 1973; Noon et al., 1998; reviewed in Maden, 1998).

Very little is known about the precise physiological importance of numerous m^5C residues present in tRNA and rRNA. In particular, it was demonstrated that the presence of m^5C_{34} at the wobble position of the anticodon loop in yeast $tRNA^{Leu}$ affects its efficiency as a suppressor in vivo (Strobel & Abelson, 1986). On the other hand, NMR and structural studies performed on yeast $tRNA^{Phe}$ and $tRNA^{Phe}$ anticodon stem-loop suggest the crucial importance of m^5C_{40} for correct spatial organization of the anticodon stem-loop and for formation of the Mg^{2+}-binding pocket (Chen et al., 1993; Lam et al., 1995; Basti et al., 1996; reviewed in Agris, 1996).

As for all other modified nucleotides present in RNA, the formation of such methylated residues is catalyzed by a family of specific RNA:methyltransferases. The activity of these enzymes with various substrate specificities can be detected in different cell extracts, provided that S-adenosyl-L-methionine, the donor of the methyl group, is added (reviewed in Garcia & Goodenough-Lashua, 1998). As far as RNA:m^5C-transferases are concerned, several attempts have already been made to purify and characterize these enzymes from different sources (Wierzbicka et al., 1975; Keith et al., 1980; Obara et al., 1982). In particular, highly enriched preparations of nucleolar RNA:m^5C-methylase from Ehrlich ascites tumor cells (Obara et al., 1982) and tRNA:m^5C-methylase from HeLa cells (Keith et al., 1980) were obtained and the activity and substrate specificity of the enzymes were tested with different RNA substrates. The molecular masses of these two enzymes were quite different (130 kDa and 72 kDa, respectively, as measured by sucrose gradient centrifugation). In both cases the purified protein fractions displayed rather broad substrate specificity and were capable of modifying a wide spectrum of RNA substrates (tRNA, rRNA, plant virus RNA, synthetic RNA copolymers containing C, but not DNA), albeit at different reaction rates. Except for tRNA, the exact location of the methylation sites was not identified. Despite this careful biochemical characterization, the genes encoding these higher eukaryotic RNA:m^5C-methyltransferases have not been identified. Only recently J. Ofengand and co-workers have succeeded in identifying the gene *rsmB* in *E. coli*, which encodes an rRNA:m^5C-methyltransferase catalyzing very specifically the formation of m^5C at position 967 in 16S rRNA (Tscherne et al., 1999).

To identify and clone genes corresponding to RNA:m^5C-methyltransferases in yeast, we used the amino acid sequence of *E. coli* rsmB protein to screen the yeast *Saccharomyces cerevisiae* genome database. Homology search using known sequences of modification enzymes from prokaryotic organisms as queries has already been successfully used for identifying different RNA:methylases and pseudouridine synthases in various bacterial and eukaryotic genomes (see, e.g., Koonin et al., 1995, 1996; Gustafsson et al., 1996). In several cases such putative identification was further confirmed by experimental verification of the enzymatic activity and substrate specificity (see, e.g., Simos et al., 1996; Becker et al., 1997a; Conrad et al., 1998; Lecointe et al., 1998; Cavaillé et al., 1999).

In this work we report the identification of the gene and the substrate specificity of tRNA:m^5C-methyltransferase from yeast *S. cerevisiae*. This enzyme catalyzes the formation of m^5C at four different positions in tRNA and therefore corresponds to a newly identified multisite-specific enzyme. Because this is the fourth identified yeast tRNA methyltransferase (after Trm1 for tRNA:$m^2{}_2G_{26}$-methylase, Trm2 for tRNA:m^5U_{54}-methylase, and Trm3 for tRNA:Gm_{18}-methylase), we propose to name the gene corresponding to yeast ORF YBL024w *TRM4* and the corresponding protein Trm4.

RESULTS

Search for tRNA:m^5C-methyltransferase gene and protein alignment

The search for tRNA-specific yeast m^5C-methyltransferase was based on the general approach described and already successfully used for the iterative search for different modification enzymes such as RNA: pseudouridine synthases and bacterial RNA:methylases (Koonin et al., 1995, 1996; Gustafsson et al., 1996). The protein sequence of the recently identified *E. coli* 16S rRNA:m^5C_{967}-methyltransferase (gene *rsmB*, also called *fmu/fmv/SUN*; SwissProt accession number P36929; Leung et al., 1998) was used as a query. The BLAST search on the complete yeast *S. cerevisiae* genome detected one protein (encoded by ORF YNL061w, also called *NOP2*) and two other ORFs (YBL024w and YNL022c) presenting significant sequence homology with *E. coli* rRNA m^5C-methylase. The yeast protein Nop2p was already partially characterized and shown to be implicated in the maturation of 26S ribosomal RNA (Hong et al., 1997), which is also consistent with its nucleolar localization in yeast (de Beus et al., 1994). The other yeast homolog (YBL024w, designated as gene *NCL1*) has also been localized in the nucleus and was found to be concentrated at the nuclear periphery. The disruption of this nonessential gene in yeast does not affect growth and ribosomal biogenesis (Wu et al., 1998).

A similar search performed on the complete nonredundant GenBank database led to selection of a group of proteins (currently 24 sequences) originating from different organisms but sharing the same conserved

sequence signatures. Although most proteins are of bacterial origin (so-called *fmv/fmu/SUN* gene family), homologs are also present in Archaea and Eukarya (both in yeast and mammals). Based on the experimental identification of m^{5}C-methyltransferase activity of *E. coli rsmB*, we assumed that most if not all of the proteins in the selected group belong to a family of RNA:m^{5}C-methyltransferases, but that their RNA substrate specificity is different.

Multiple sequence alignment of putative RNA:m^{5}C-methyltransferases (see Fig. 1) was constructed using ClustalW (Thompson et al., 1994) and MACAW (Lawrence et al., 1993) software and further refined manually. Despite very important differences in size [ranging from 225 amino acids for the shortest family member (fmv protein from *Vibrio alginolyticus*) to 855 amino acids for human proliferating-cell nucleolar antigen p120 (encoded by gene *NOL1*)], all proteins share several highly conserved sequence blocks (numbered I to V on Fig. 1). It is noteworthy that all eubacterial and archaeal proteins (except *V. alginolyticus* fmv) have rather similar lengths (about 300 amino acids). Also, the conserved blocks are located at similar positions inside the sequences. In contrast, eukaryotic homologs are considerably longer and vary in respect to positions of the conserved domains.

Close inspection of the conserved regions (Fig. 1, blocks I–V) allowed us to detect the putative Adomet-binding motifs present in all analyzed sequences (Fig. 1, blocks II and III). Compared to Adomet-binding motifs detected in other methyltransferases of different substrate specificity (Kagan & Clarke, 1994; Koonin, 1994; see Fig. 1, bottom), the putative motifs in m^{5}C-methyltransferases differ by high conservation of alanine residues (A, underlined on Fig. 1 bottom) in Block II (instead of glycine, G) and by the presence of highly conserved proline-cysteine (PC, underlined) dipeptide in Block III (see also Discussion). Otherwise the general distribution of hydrophobic amino acids in motifs and locations of universally conserved aspartic acid (D) and glycine (G) residues in motifs I and II are well preserved.

In contrast to *E. coli*, where m^{5}C was found in rRNA (Smith et al., 1992), but not in tRNA (Sprinzl et al., 1998), in yeast this methylated cytosine is present in both tRNA (four positions, see below) and in rRNA (at least two residues in 26S rRNA, the exact positions of which are not yet known). One could therefore expect that at least one of the detected yeast ORFs (YBL024w, YNL061w, or YNL022c, underlined on Fig. 1 top) corresponds to a RNA:m^{5}C-methyltransferase specific for tRNA. Taking into account the nucleolar localization of Nop2p (encoded by ORF YNL061w) and the values of the BLAST score for sequence homology with *E. coli* rsmB protein, we hypothesized that the protein encoded by yeast *S. cerevisiae* ORF YBL024w was the best candidate for being a tRNA:m^{5}C-methyltransferase.

Cloning of ORF YBL024w and expression of the protein in *E. coli*

To identify the activity and substrate specificity of putative RNA:m^{5}C-methyltransferase encoded by yeast ORF YBL024w, the corresponding sequence (2,055 bp) was amplified by PCR and cloned in *E. coli* expression vector pET28b downstream to a His$_6$-tag and a thrombin cleavage site (MGSSHHHHHHSSGLVPRGSH MAS). The resulting construct allowed the expression of the recombinant protein upon IPTG induction in *E. coli* BL21 (DE3) cells.

Detection of tRNA:m^{5}C-methyltransferase activity in vivo

To detect the activity of the expressed recombinant yeast methyltransferase, we analyzed first the presence of the modified nucleotide m^{5}C in bulk *E. coli* tRNA extracted from induced pET28b/YBL024w-containing cells. Naturally occurring *E. coli* tRNAs do not contain m^{5}C, but, as was demonstrated previously, these tRNAs can serve as substrates for eukaryotic tRNA:m^{5}C-methylases (see Munns & Sims, 1975; Wierzbicka et al., 1975; Keith et al., 1980). Control *E. coli* cells used for comparison were transformed by the pET28b vector. Analysis of the tRNA modification pattern was performed by reversed-phase high performance liquid chromatography (HPLC) separation of nucleosides. The resulting profiles (Fig. 2) clearly show the appearance of the peak corresponding to m^{5}C (indicated by an arrow on Fig. 2B) in the case of tRNA derived from pET28b/YBL024w-transformed strain. The presence of m^{5}C in total tRNA extracted from pET28b/YBL024w-transformed *E. coli* strain was also confirmed by postlabeling (data not shown). These data served as a demonstration that recombinant Ybl024p indeed represents an active tRNA:m^{5}C-methylase capable of modifying bulk *E. coli* tRNA in vivo.

Hyperexpression of the Ybl024 protein in *E. coli* and its purification

The expression the recombinant protein (Ybl024p) was performed in *E. coli* BL21 (DE3). Analysis of the expressed products by gel-electrophoresis in denaturing conditions and by Western blotting with anti-His$_6$ antibodies showed only a very small amount of Ybl024p (data not shown). Because the ORF YBL024w contains as many as 26 arginine codons (AGA) out of a total of 36 (72%) and some of them even occur in tandem, we suspected that the limited supply of the minor *E. coli* tRNA$^{Arg}_{(UCU)}$ decoding these AGA codons was the main reason for the low expression of the recombinant protein. To overcome this problem, the pET28b/YBL024w-transformed cells were cotransformed by the plasmid pDC952 bearing *E. coli*

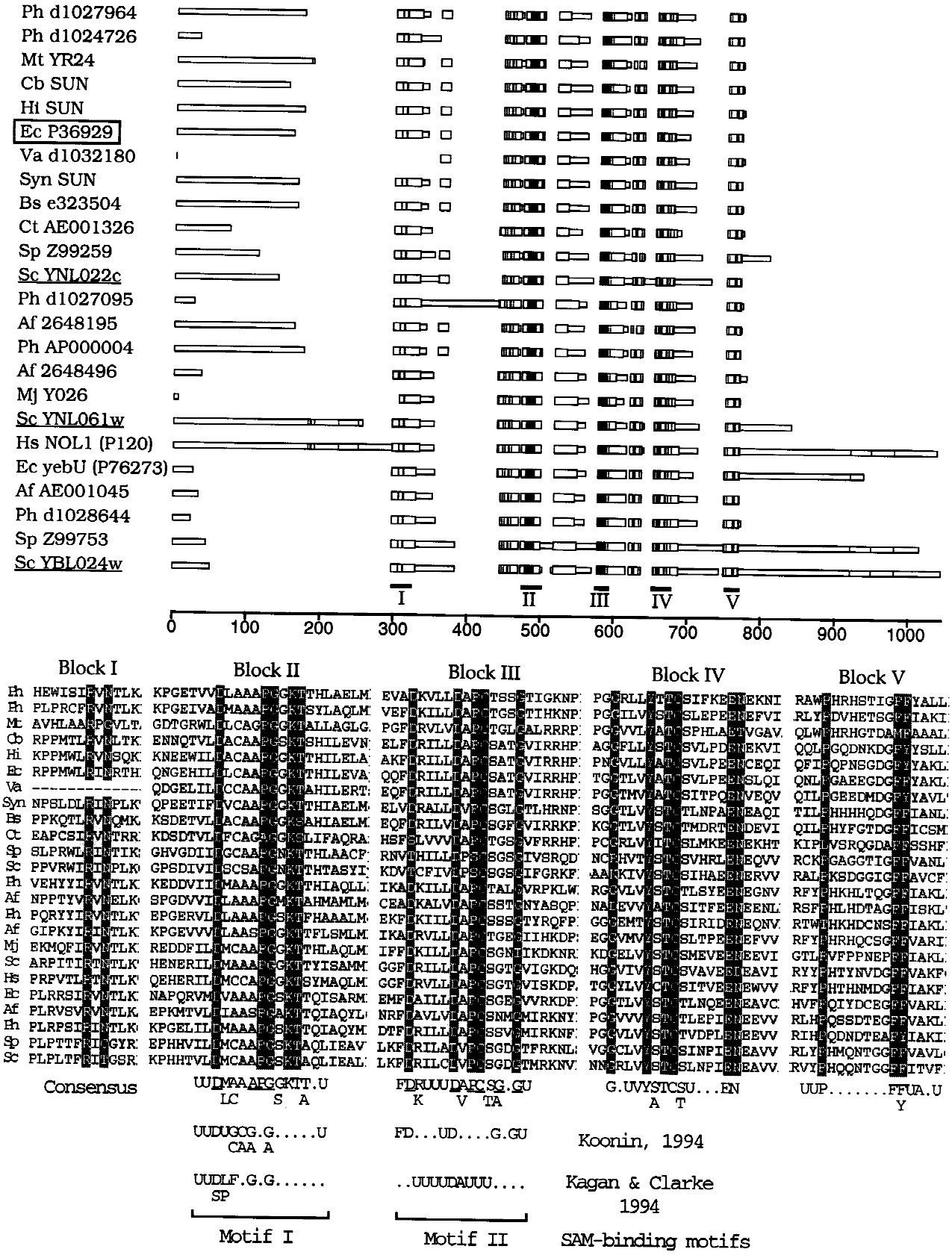

FIGURE 1. (*Legend on facing page.*)

$tRNA^{Arg}_{(UCU)}$ (gene *argU*). The IPTG-induction tests demonstrated indeed that the presence of this tRNA permits an increase of the expression level of Ybl024p by a factor of at least 20. Expression conditions were further optimized by varying the composition of the induction medium (LB or MM9 media) and time (from 30 min to 4 h). The results of such optimization are presented in Figure 3 (left part). The protein with a molecular mass of about 82 kDa already appears on the denaturing polyacrylamide gel after 30 min of IPTG induction in minimal medium (MM9) and accumulates to high amounts after 2–3 h. Further increase of induction time (up to 4 h) does not significantly improve the expression level, but rather leads to accumulation of degradation products, as verified using Western blotting with anti-His_6 antibodies (not shown).

The purification of recombinant Ybl024p from the *E. coli* extract was achieved by batch affinity adsorption on a Ni^{2+}-NTA-agarose matrix followed by washing with 20 mM imidazol and elution with 300 mM imidazol. Analysis of the recovered protein fraction is presented in Figure 3 (right part). The affinity-purified protein appears almost homogeneous upon electrophoresis in the denaturing conditions, and most impurities visible at high load represent the His_6-tag containing degradation intermediates (as detected by Western blotting using anti-His_6 antibodies; not shown). The purified recombinant protein migrates with an apparent molecular mass of 82.2 kDa that correlates well with the value of 80.2 kDa calculated from amino acid sequence, including His_6- and thrombin cleavage sequences.

Detection of tRNA:m^{5}C-methyltransferase activity in vitro

The survey of the most recent release of the tRNA DataBank (Sprinzl et al., 1998) indicates that m^{5}C is found at four different positions in yeast tRNAs (positions 34, 40, 48, and 49; Fig. 4A). However, the frequency of modification depends on the cytosine position in tRNA. At positions 34 and 40, m^{5}C is found only in two yeast tRNAs ($tRNA^{Leu}_{(CUA)}$ and $tRNA^{Phe}_{(GAA)}$, respectively), whereas most other elongator yeast tRNAs bear either m^5C_{48} or m^5C_{49}, but never both in the same tRNA molecule.

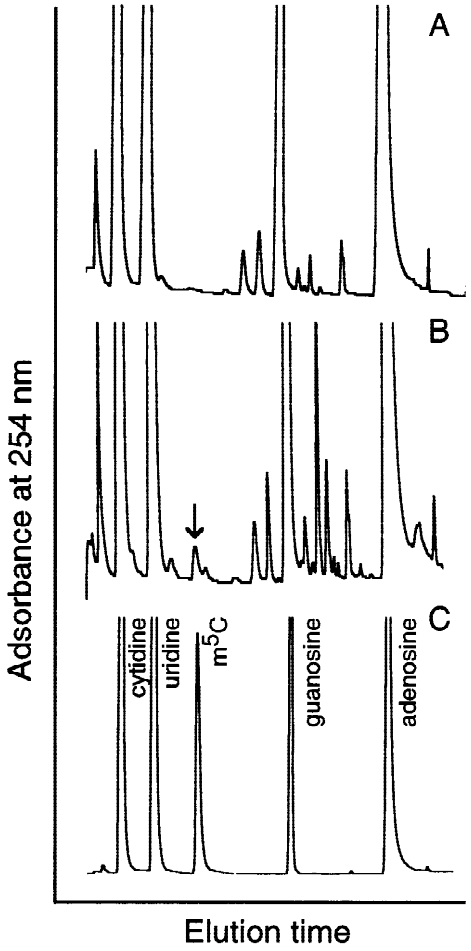

FIGURE 2. Detection of m^{5}C in bulk tRNA extracted from *E. coli* BL21(DE3) transformed by pET28b/YBL024w. The total tRNA fraction was completely hydrolyzed by nuclease P1 and the resulting nucleotides dephosphorylated by calf intestine alkaline phosphatase. Analysis of nucleosides was performed by reverse-phase chromatography on a Lichrosphere 100RP18 (C_{18}) (250 × 4.6 mm i.d., Bischoff, Germany) column in the conditions described by Gehrke & Kuo (1990). Chromatographic profiles of nucleosides derived from control pET28b-transformed *E. coli* BL21(DE3) (**A**) and from *E. coli* BL21(DE3) transformed by pET28b/YBL024w (**B**). Separation of a test nucleoside mixture (U, C, m^{5}C, G, and A) is presented in **C**. The position of m^{5}C is indicated by an arrow (**B**).

The enzymatic formation of m^5C_{48} and m^5C_{49} can be readily detected using yeast extracts and various in vitro prepared yeast tRNA (Becker et al., 1997b; Jiang et al., 1997). In contrast, because the formation of both

FIGURE 1. Sequence alignment of RNA:m^{5}C-methyltransferases from different organisms. The following abbreviations of organisms names are used. Ph: *Pyrococcus horikoshii*; Mt: *Mycobacterium tuberculosis*; Cb: *Coxiella burnetii*; Hi: *Hemophilus influenzae*; Ec: *Escherichia coli*; Va: *Vibrio alginolyticus*; Syn: *Synechocystis sp.*; Bs: *Bacillus subtilis*; Ct: *Chlamidia trachomatis*; Sp: *Schizosaccharomyces pombe*; Sc: *Saccharomyces cerevisiae*; Af: *Archaeoglobus fulgidus*; Mj: *Methanococcus jannaschii*; and Hs: *Homo sapiens*. The abbreviated name of the organism is followed by the accession number in GenBank or SwissProt databases. The scale in amino acid residues is also presented. Identical and conserved residues are shaded. Two putative AdoMet-binding motifs and their consensus amino acid sequences are shown at the bottom. A one-letter code for each amino acid was used, except in the listing of consensus sequences, where the hydrophobic amino acids I, L, V, M, F, and A were collectively designated by U. The upper line of the consensus motifs corresponds to those defined in this article, whereas the second and third lines correspond to the motifs defined by Koonin (1994) and Kagan & Clarke (1994), respectively. Ribosomal RNA m^5C_{967}-methyltransferase from *E. coli* is boxed in the upper part of the figure. Three yeast ORF are underlined.

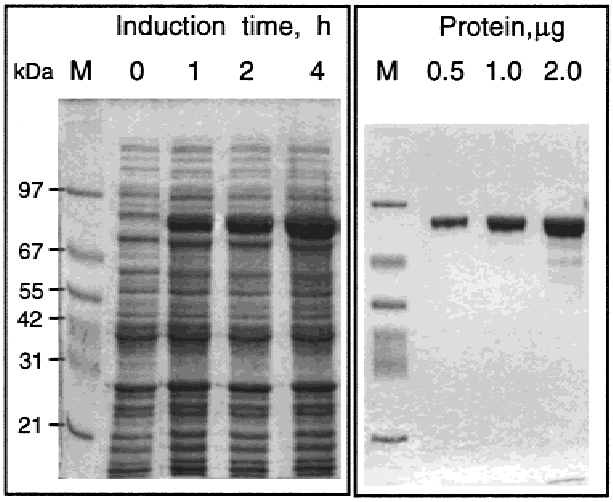

FIGURE 3. Expression of yeast protein Ybl024p in *E. coli* BL21 (DE3). IPTG-induced cells were harvested by centrifugation, lysed in the presence of 6% sodium dodecyl sulfate (SDS), and the extract was electrophoresed on 8% denaturing polyacrylamide gel. The time-course of IPTG induction is presented on the left. The mid-range molecular mass markers (Promega, USA) were loaded into lane M. The purified recombinant Ybl024p is presented on the right. Different amounts (as indicated) of the Ni^{2+}-NTA-agarose purified recombinant protein were applied on the gel.

m^5C_{34} and m^5C_{40} is a strictly intron-dependent process and takes place only in the intron-containing tRNA precursors (Strobel & Abelson, 1986; Jiang et al., 1997; for review see Grosjean et al., 1997), these modifications can be detected in vitro only using intron-containing $tRNA^{Leu}$ and $tRNA^{Phe}$ precursors. However, the corresponding minisubstrates composed of the anticodon stem-loop extended by the intron were shown to be efficient minisubstrates for the tRNA:m^5C-methyltransferases present in the yeast extract (Grosjean et al., 1997; Jiang et al., 1997). Despite this finding, the methylases catalyzing m^5C formation at these four positions in yeast tRNAs were not yet characterized.

The cloverleaf presentation of yeast RNA substrates used in this study to detect the activity of the purified recombinant Ybl024p is shown in Figure 4B–H. Two RNA substrates ($tRNA^{Phe}_{(GAA)}$ and $tRNA^{Asp}_{(GUC)}$) could test the formation of m^5C_{49}; three other tRNAs ($tRNA^{Tyr}_{(GUA)}$, $tRNA^{Ser}_{(AGA)}$, and $tRNA^{Ile}_{(UAU)}$) could test the formation of m^5C_{48}, and two minisubstrates (mini-$tRNA^{Leu}$ and mini-$tRNA^{Phe}$) could test the formation of m^5C_{34} and m^5C_{40}, respectively.

The activity of recombinant Ybl024p was first tested using radioactive [^{3}H]AdoMet and in vitro prepared non-radioactive transcripts of yeast $tRNA^{Asp}$, $tRNA^{Phe}$, and two minisubstrates (mini-$tRNA^{Leu}$ and mini-$tRNA^{Phe}$). The results presented in Figure 5 clearly indicate that all four RNA substrates allow detectable incorporation of [^{3}H]-methyl group from AdoMet into RNA transcripts catalyzed by the recombinant Ybl024p. This demonstrates that Ybl024p is indeed a AdoMet-dependent methyltransferase acting on tRNA.

Identification of the methylated cytosine residues and specificity of recombinant Ybl024p in vitro

The activity and specificity of recombinant tRNA:m^5C-methyltransferase were further confirmed by using [α-^{32}P]radiolabeled RNA substrates. This technique also confirmed that the methylated product formed upon incubation with recombinant Ybl024p is indeed m^5C. Identification was performed by the "nearest-neighbor" analysis. Depending on the nature of the nucleotide 3′ adjacent to the cytosine to be modified (see Fig. 4), the RNA transcripts were radiolabeled with either [α-^{32}P]GTP or [α-^{32}P]UTP. The transcripts of $tRNA^{Tyr}$, $tRNA^{Ser}$, and $tRNA^{Ile}$ were used to detect the formation of m^5C_{48}, and $tRNA^{Asp}$ and $tRNA^{Phe}$ were used for detection of m^5C_{49}. Likewise, minisubstrates mini-$tRNA^{Leu}$ and mini-$tRNA^{Phe}$ were used for detection of m^5C_{34} and m^5C_{40} respectively. The results of two-dimensional tlc analysis of nucleosides 3′-monophosphates derived from in vitro-modified transcripts are presented in Figure 6. Similar results were obtained for all RNA substrates used, but only representative examples of m^5Cp derived from positions 48, 49, 34, and 40 are shown. Control incubations done using control (transformed by pET28b) *E. coli* BL21(DE3) extract confirmed the absence of the corresponding activity in *E. coli* (data not shown) and thus excluded the possible contamination of recombinant Ybl024p by endogenous tRNA:m^5C-methyltransferase. The corresponding time-courses for all substrates are presented on Figure 7. All tests were performed at identical concentrations of the enzyme and similar concentrations of the transcripts. The modification rate is different for the different RNA substrates (see also Fig. 5); however all substrates are modified to more than 0.7 mol/mol after 1 h incubation. The lower plateau levels of modification observed in the case of minisubstrates of $tRNA^{Leu}$ and $tRNA^{Phe}$ (Fig. 7B) most probably result from inappropriate folding of a fraction of these molecules under the conditions of the assay.

Taken together our results allow us to conclude that the yeast ORF YBL024w indeed encodes AdoMet-dependent tRNA-specific m^5C-methyltransferase and this enzyme is capable of modifying cytosine at positions 34, 40, 48, and 49. According to conventional nomenclature of yeast tRNA methyltransferases, we suggest assigning the name *TRM4* (the fourth tRNA methyltransferase identified in yeast) to ORF YBL024w.

tRNA:m^5C-methyltransferase activity is missing in the extract of ΔYBL024w yeast strain

To verify the specificity of tRNA:m^5C-methyltransferase (Trm4) in vivo we prepared a yeast strain in which the corresponding ORF was entirely replaced by the marker

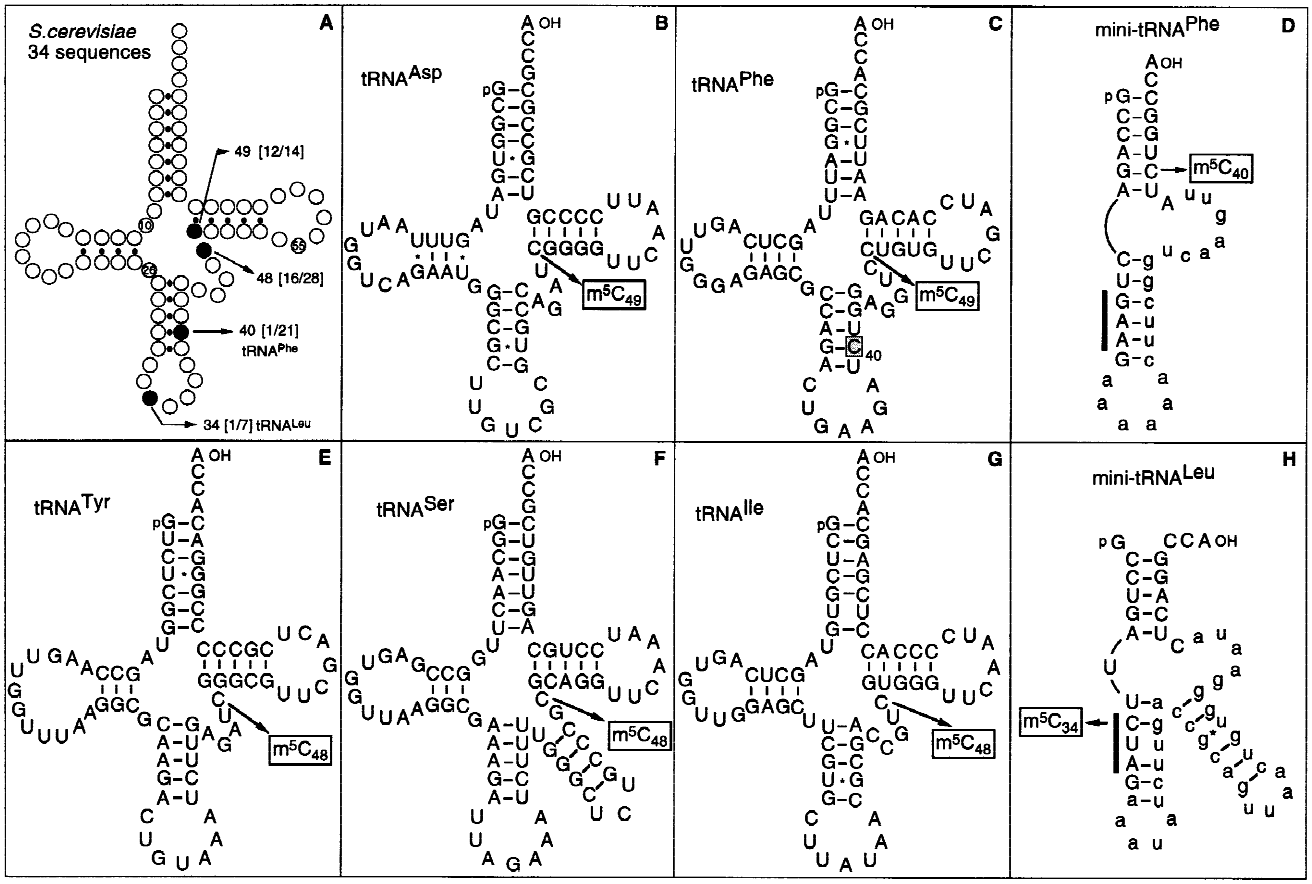

FIGURE 4. Occurrence of 5-methylcytidines in the compiled nucleotide sequences of yeast tRNAs (**A**) in the secondary structures and sequences of T7 transcripts corresponding to specific yeast tRNAs (**B**–**C**, **E**–**G**) and in intron-containing mini-tRNAs comprising the anticodon stem-loop (capital letters) and the intron (small letters) (**D** and **H**) used in this study. Numbers in the cloverleaf correspond to location in tRNA. Numbers in brackets in **A** indicate the frequency of m^{5}C modification. The first number in each bracket indicates the frequency of occurrence of m^{5}C; the second number corresponds to the frequency of the parent nucleotide (Cytosine). The thick bar in **D** and **H** indicates the anticodon sequence in tRNA minisubstrates. The second potential site of m^{5}C methylation (pos 40) in intronless tRNA^{Phe} is shaded.

conferring Geneticin (G_{418})-resistance in yeast. As reported before, the disrupted ΔYBL024w yeast strain remains viable and shows no apparent growth phenotype (Wu et al., 1998).

Direct implication of Trm4 in modification of yeast tRNA in vivo was verified by analysis of m^{5}C content in the fraction of total tRNA extracted from the wild-type and ΔYBL024w-disrupted strains. Total tRNA purified by ion-exchange chromatography on Mono Q (to remove yeast rRNA that also contain m^{5}C) was completely hydrolyzed by the nuclease P1 and dephosphorylated to nucleosides by alkaline phosphatase as described in Materials and Methods. The results of HPLC analysis are given in Figure 8. The peak corresponding to m^{5}C is well separated from the adjacent m^{1}A peak in the hydrolysate of the wild-type yeast tRNA (Fig. 8B). The disruption of ΔYBL024w leads to complete disappearance of an m^{5}C peak on the profile (Fig. 8C), whereas the relative proportion of other modified nucleotides remains constant. These

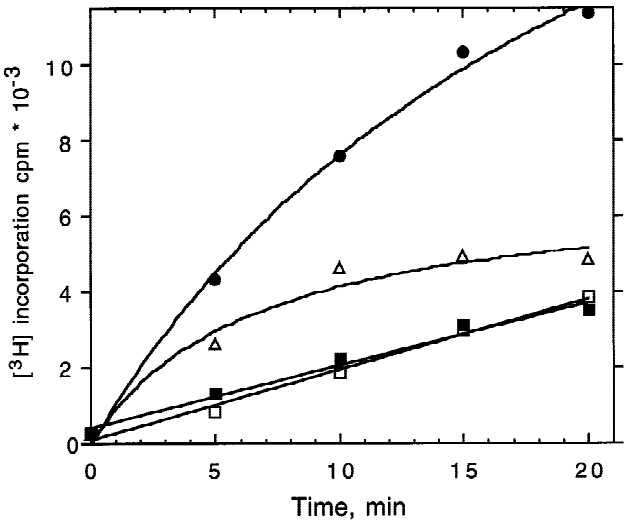

FIGURE 5. Time course of [^{3}H]methylation of different RNA transcripts upon incubation with purified recombinant Ybl024p. Yeast tRNA^{Asp} (filled circles), yeast tRNA^{Phe} (filled squares), mini-tRNA^{Leu} (open squares), and mini-tRNA^{Phe} (open triangles).

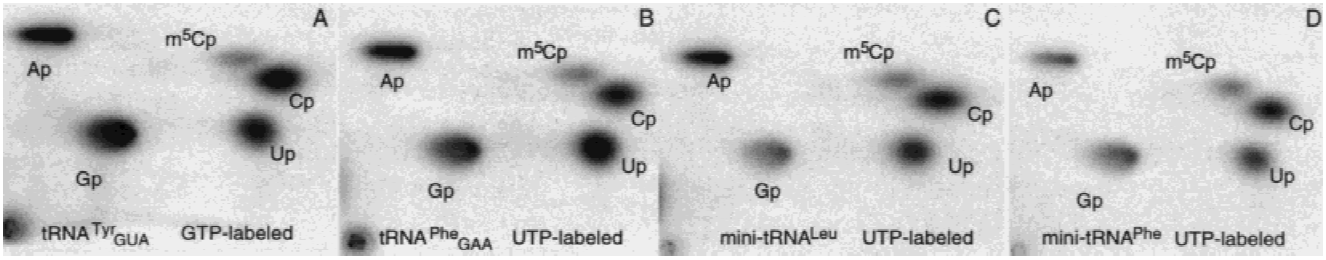

FIGURE 6. Formation of m^5C in different RNA substrates upon the incubation with purified Ybl024p. The figure shows selected autoradiograms of two-dimensional tlc plates of 3′-monophosphate nucleotides obtained after complete digestion of the radiolabeled RNA with RNase T2 (nearest neighbor analysis). All transcripts were uniformly radiolabeled by [α-^{32}P]GTP or [α-^{32}P]UTP, as indicated on the corresponding panels. The chromatographic system (N/R) has been described previously (Auxilien et al., 1996). Identification of nucleotides was made by comparison with references maps, as in Keith (1995).

results demonstrate that Trm4 (encoded by YBL024w) is responsible for m^5C methylation of tRNA at major sites (positions 48 and 49).

To confirm the implication of Trm4 in formation of unique m^5C residues at positions 34 and 40 in $tRNA^{Leu}$ and $tRNA^{Phe}$, we compared the tRNA:m^5C-methyltransferase activity in the extracts prepared from wild-type parental strain (*S. cerevisiae* W303) and from YBL024w-disruptant. The results of in vitro tests are presented in Figure 9. The m^5C methylation of the cytosine residues at positions 34 (in the transcript of mini-$tRNA^{Leu}$), 40 and 49 (in pre-$tRNA^{Phe}$), 48 (in $tRNA^{Ser}$), and 49 (in $tRNA^{Asp}$) can be readily detected in the extract of wild-type yeasts. In contrast, no formation of m^5C residues in all tested substrates was detected when the extract of ΔYBL024w-disrupted strain was used. These results confirm the data obtained in vitro with the recombinant His_6-Ybl024p and allow us to conclude that tRNA:m^5C-methyltransferase Trm4 (encoded by ORF YBL024w) is the only enzyme that is responsible for the formation of m^5C residues in all four positions where this modified nucleotide is found in mature yeast tRNA.

DISCUSSION

In this article we describe the cloning of a yeast ORF YBL024w, as well as the purification and identification of the enzyme activity of the corresponding gene product. The gene was selected on the basis of its amino acid sequence homology with a recently identified rRNA:m^5C-methyltransferase from *E. coli* (Tscherne et al., 1999). Such a homology-based approach is now becoming a classical way to pinpoint genes belonging to a given family of proteins or enzymes with identical or different specificity, to compare them, and to deduce common sequence motifs as well as possible evolutionary interrelationships. The availability of complete genomic sequences for an increasing number of very different organisms is of particular advantage for that purpose.

Yeast protein Trm4 (Ybl024p) catalyzes m^5C formation at four distinct sites in tRNA

Modified nucleotide m^5C is found at four different positions in yeast tRNA (Fig. 4A). The formation of m^5C at positions 48 and 49 does not depend on the presence of an intron (Grosjean et al., 1997) or even on the correct global three-dimensional tRNA structure (Nishikura et al., 1982; Grosjean et al., 1996; Brulé et al., 1998). In contrast, formation of m^5C_{34} and m^5C_{40} is strictly intron-dependent and both the sequence and the folding of the intron are important for correct recognition (Strobel & Abelson, 1986; Jiang et al., 1997). The discovery that the enzyme Trm4 is capable of modifying all four potential positions in yeast tRNA indicates that a single protein may account for formation of all m^5C present in the different yeast tRNAs. The RNA recognition mode of this protein obviously does not rely on global three-dimensional tRNA structure, as confirmed by efficient modification of tRNA minisubstrates completely lacking the L-shaped tRNA structure. Moreover, the experiments performed by Strobel and Abelson (1986) strongly indicate that the correct base-pairing in the intron structure and the presence of the complete intron sequence is crucial for modification of m^5C_{34} in pre-$tRNA^{Leu}$. Most probably this newly identified multisite-specific yeast tRNA:m^5C-methyltransferase recognizes only a small structural domain present both in tRNA and minisubstrate molecules.

Taken together, the results obtained in vitro (using the recombinant His_6-Trm4) and in vivo (using the corresponding yeast disrupted strain) clearly demonstrate that yeast tRNA:m^5C-methyltransferase Trm4 is the only methyltransferase in yeast that is required and sufficient for m^5C methylation of all potential sites in tRNA. No other protein with similar activity acting on tRNAs is present in yeast.

Three groups of RNA:m^5C-methyltransferases

Sequence comparison of 24 putative RNA:m^5C-methyltransferases (commonly referred as the proteins of the

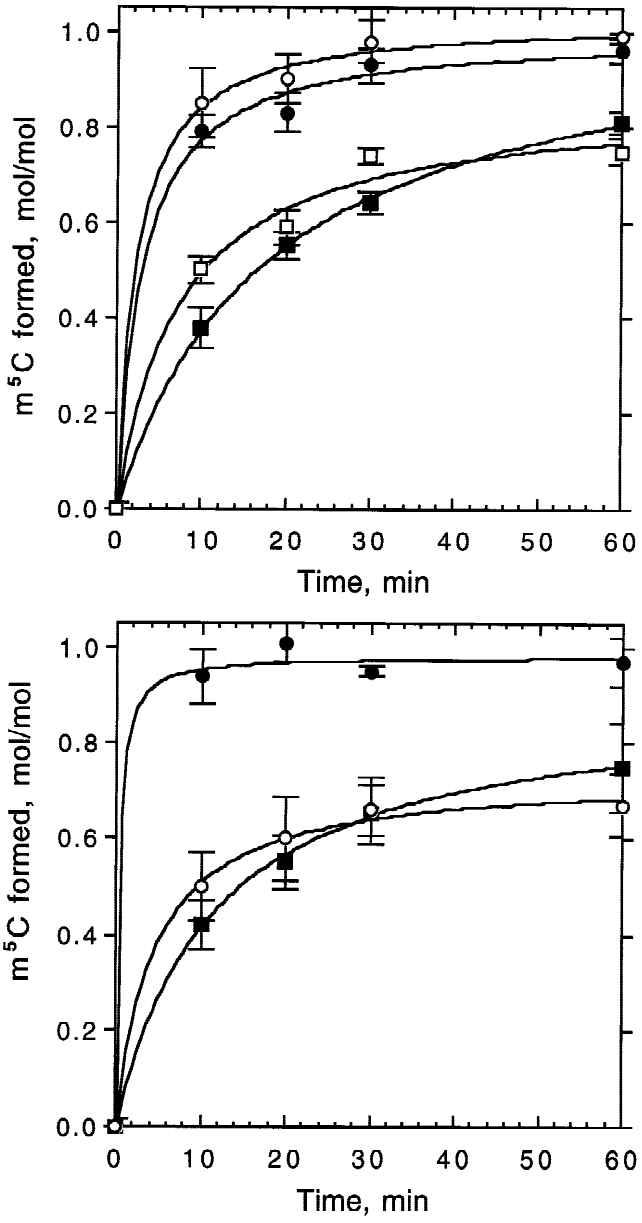

FIGURE 7. Time course for the formation of m^{5}C in different tRNAs and tRNA minisubstrates. The upper panel presents the kinetics of modification for tRNA^{Asp} (filled circles), tRNA^{Phe} (filled squares), tRNA^{Tyr} (open circles), and tRNA^{Ser} (open squares). The bottom panel shows the modification of tRNA^{Ile} (filled circles), mini-tRNA^{Leu} (filled squares), and mini-tRNA^{Phe} (open circles). Values were calculated from quantification of the relative radioactivity in the corresponding spots as detected in Figure 5 for different incubation times.

fmv/SUN/P120 family; Fig. 1) allows us to classify these proteins in three subclasses that probably differ by their RNA specificity. The phylogenetic tree was constructed taking into account only highly conserved core parts of proteins (Fig. 10). Most bacterial homologs form the group of putative rRNA:m^{5}C-methyltransferases (group I) together with *E. coli* protein P36929, the specificity of which was experimentally determined, whereas the majority of the eukaryotic proteins (except two, Sc YNL022c and Sp Z99259) form two distinct subclasses (groups II and III). One of the *E. coli* proteins (encoded by the gene *yebU*–P76273) clearly falls into group III, containing the yeast tRNA:m^{5}C-methyltransferase (Sc YBL024w). However, *E. coli* does not contain m^{5}C in any tRNA sequenced so far and no corresponding activity was detected in a *E. coli* extract using heterologous yeast tRNA transcripts (data not shown). Thus it is tempting to speculate that the *E. coli* yebU protein represents a m^{5}C-methyltransferase acting on RNA other that tRNA, possibly catalyzing the formation of m^{5}C$_{1407}$ in 16S and/or m^{5}C$_{1962}$ in 23S rRNA.

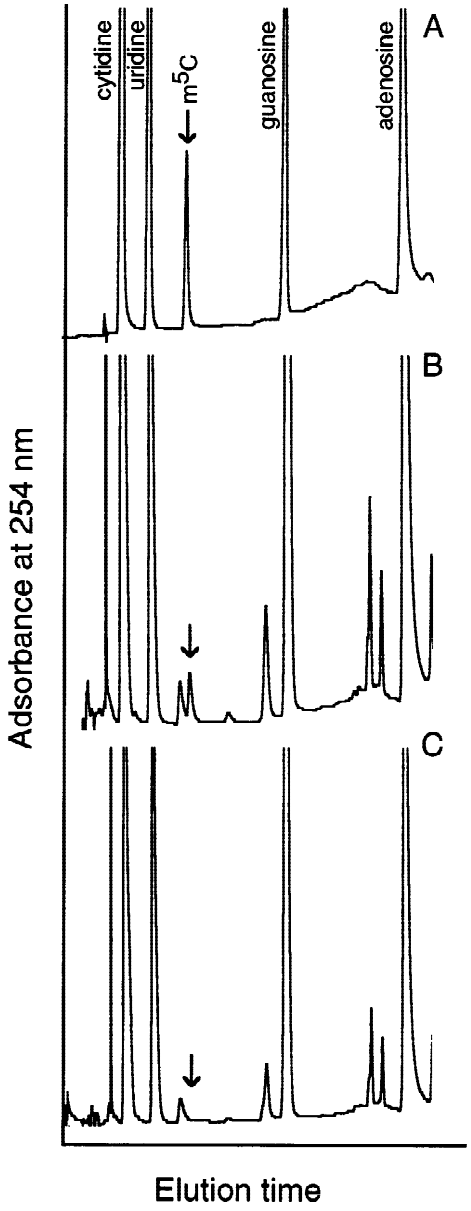

FIGURE 8. The absence of m^{5}C in bulk tRNA extracted from disrupted ΔYBL024w yeast strain. The conditions of tRNA hydrolysis and reverse-phase chromatography are described in the legend of Figure 2. Separation of a test nucleoside mixture (U, C, m^{5}C, G, and A) is presented in **A**. Chromatographic profiles of nucleosides derived from control yeast strain (**B**) and from disrupted ΔYBL024w yeast strain (**C**). The position of m^{5}C is indicated by an arrow.

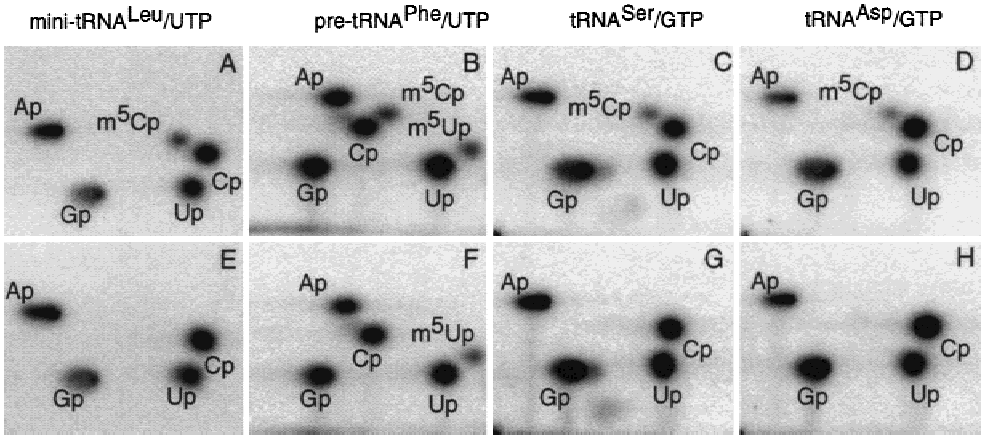

FIGURE 9. Formation of m^{5}C in different RNA substrates upon the incubation with the extract of wild-type and ΔYBL024-disrupted yeast strains. Radiolabeled transcripts were incubated for 90 min at 30 °C in the presence of S100 extract of wild-type (upper row) or mutant yeast strain (bottom row). The figure shows the autoradiograms of two-dimensional tlc plates of 3′-monophosphate nucleotides obtained after complete digestion of the radiolabeled RNA with RNase T2 (nearest neighbor analysis). The type of [α-^{32}P]NTP used for labeling is indicated on each panel. The conditions of chromatographic separation and the identification of nucleotides are described in the legend of Figure 6. To separate the spots of m^{5}C and m^{7}G formed in pre-tRNA^{Phe}, the N/N chromatographic system (Auxilien et al., 1996) was used.

The genome of yeast *S. cerevisiae* contains three proteins of the family, each falling into one of the three distinct subgroups. The protein from group III (encoded by ORF YBL024w) is responsible for cytosine conversion to m^{5}C in tRNA and tRNA precursors, as demonstrated in this work. In agreement with this identification, Ybl024p was found at the nuclear periphery but apparently is not colocalized with the

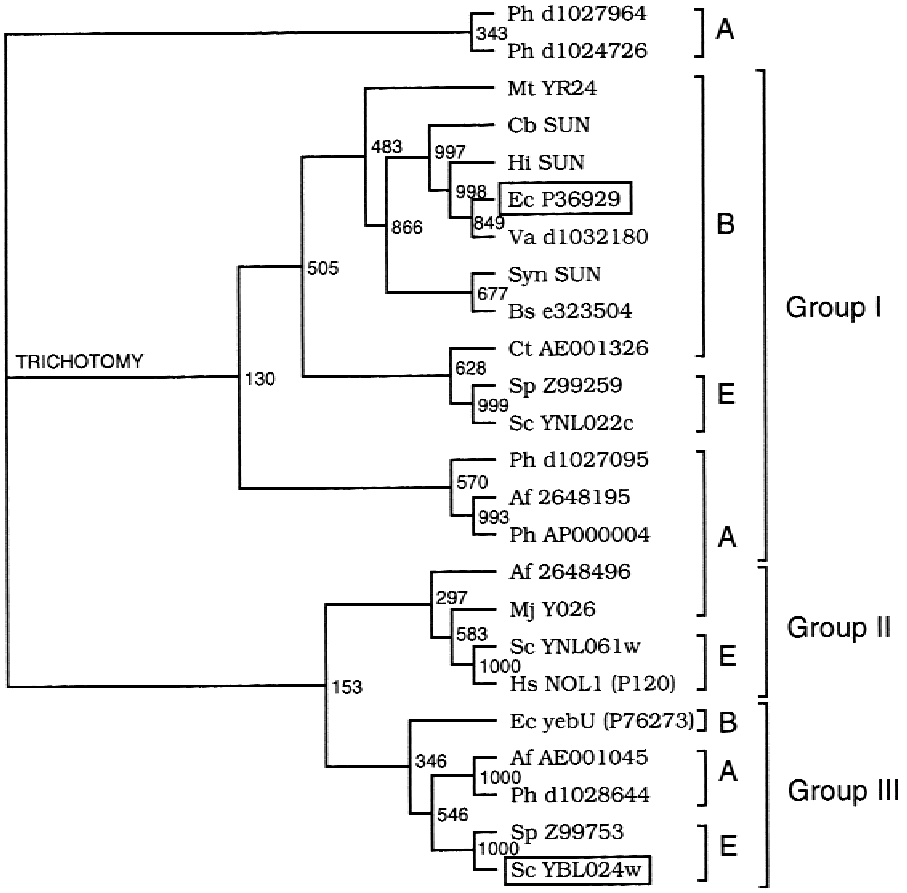

FIGURE 10. Bootstrapped phylogenetic tree of putative RNA:m^{5}C-methyltransferases. The tree was constructed using ClustalW software and the alignment of the core part of proteins excluding positions with gaps. Abbreviations of the organism names and accession numbers are as in Figure 1. Capital letters B, E and A designate Bacteria, Eukarya, and Archaea, respectively. *E. coli* site-specific rRNA:m^{5}C$_{967}$-methyltransferase (Ec P36929) and *S. cerevisiae* multisite-specific tRNA:m^{5}C-methyltransferase (Sc YBL024w) are boxed. Numbers show the percent occurrence of nodes in 1,000 bootstrap replications.

nuclear pores (Wu et al., 1998). The two other proteins [the gene products of ORFs YNL022c and YNL061w, also called *NOP2* (Hong et al., 1997)] in groups I and II may represent site-specific yeast rRNA:m^{5}C-methyltransferases. Recently it was shown that the replacement of universally conserved cysteine residue in PC sequence (Adomet-binding site, Block III, see Fig. 1) of Nop2p abolishes the functional complementation in yeast (King et al., 1999). As PC sequence is a part of Adomet-binding site, this observation supports the hypothesis that Nop2p possesses m^{5}C-methyltransferase activity. This putative identification of Nop2p as rRNA:m5C-methyltransferase is also consistent with the nucleolar localization of Nop2p (de Beus et al., 1994) and its implication in rRNA maturation (Hong et al., 1997). A very similar situation was already observed for two other rRNA modification enzymes (Dim1 methyltransferase and Cbf5 pseudouridine synthase), which are also indispensable as factors of rRNA maturation in yeast (Lafontaine et al., 1995, 1998).

The results of a search for putative RNA:m^{5}C-methyltransferases from different Archaea are noteworthy. The complete genomic sequences of four representatives of this life domain are now accessible. A BLAST search detected five homologous, but distinct proteins in *Pyrococcus horikoshii*, three in *Archaeoglobus fulgidus,* and only one in *Methanococcus jannaschii*. No corresponding sequences have been detected in the complete genome of *Methanobacterium thermoautotrophicum*. Unfortunately, information on the presence of m^{5}C in tRNA from these organisms remains extremely limited. Indeed, only two tRNA species from *M. thermoautotrophicum* have been sequenced and none contains m^{5}C at either position 48 or 49, although unmodified cytosine is present (Gu et al., 1984). Inspection of tRNA sequences from other Archaea reveals that m^{5}C is in fact present at several positions in tRNA (positions 39, 40, 48, 49, 50, and 51) (Gupta, 1984; Auffinger & Westhof, 1998). Taken together these data may indicate that the corresponding tRNA:m^{5}C-methyltransferase(s) may be present in certain Archaeal organisms while totally absent in others. Moreover, m^{5}C residues were detected in 16S and 23S rRNAs from *Sulfolobus solfataricus* (Noon et al., 1998), supporting the idea that rRNA-specific m^{5}C-methyltransferases exist at least in this archaeon.

Lastly, the genomes of the most extensively studied higher eukaryotes (mouse and human) contain proteins of the family that are homologous to yeast YNL061w (*NOP2*) (Group II, see Fig. 10). More extensive search using the current release of the human EST (Expressed Sequence Tags) database also detected homologous sequences from the other two groups (Group I and Group III). Two human ESTs (EST21244, accession number AA319080 and zt19h10.r1, accession number AA283855) display similarity to yeast YNL022c, and human EST nz95e12.s1 (accession number AA732566) may be related to yeast YBL024w. This clearly indicates the presence of enzymes from all three families of RNA:m^{5}C-methyltransferases in higher eukaryotic cells.

Sequence homology with DNA:m^{5}C-methyltransferases (MTases)

The enzymatic formation of modified nucleoside m^{5}C in RNA should have some points in common with m^{5}C methylation in DNA. The enzymes (MTases) acting on DNA have been detected in most living organisms, and the mechanism of DNA methylation was rather well studied by different approaches (reviewed in Adams, 1995; Cheng, 1995; Roberts, 1995; Cheng & Blumenthal, 1996). In addition, the crystal structures of M.*HpaI* DNA:m^{5}C-methyltransferase in binary complex with AdoMet and in ternary complex with AdoMet and a substrate analog have been resolved (Cheng et al., 1993; Klimasauskas et al., 1994). Despite very similar substrates (polynucleotide chain RNA/DNA and AdoMet) and a common reaction product (5-methylcytidine), the two enzymatic families (DNA and RNA m^{5}C-methyltransferases) display very little homology at the level of amino acid sequences. At least five highly conserved sequence motifs have been detected in DNA:m^{5}C-methylases (Posfai et al., 1989; Malone et al., 1995; O'Gara et al., 1995), two of them corresponding, in fact, to common Adomet-binding motifs. Despite great divergence in amino acid sequences, both DNA and RNA:m^{5}C-methyltransferases share the highly conserved dipeptide sequence (PC) in the second Adomet-binding motif (Block III on Fig. 1), whereas this dipeptide (PC) is not obligatorily conserved in other Adomet-binding methylases. In the case of DNA:m^{5}C-methylases, the PC sequence is part of the enzyme active site, with the cysteine residue binding covalently to the target cytosine (Wu & Santi, 1987; for review see Adams, 1995; Cheng, 1995). Recently, the site-directed mutagenesis of yeast Nop2 confirmed the crucial importance of the cysteine residue in Block III for functional complementation in vivo (King et al., 1999).

Multisite-specific enzymes in eukaryotes

The multisite-specific tRNA:m^{5}C-methyltransferase (Trm4p/Ybl024p) obviously belongs to the recently discovered class of multisite-specific RNA modification enzymes (Simos et al., 1996; Motorin et al., 1998). The first member of this class, yeast RNA:pseudouridine synthase 1 (Pus1) is capable of catalyzing the formation of pseudouridine at eight distinct sites in tRNA and in one site in U2 snRNA in yeast (Motorin et al., 1998; Massenet et al., 1999). The discovery of multisite-specific tRNA:m^{5}C-methylase (Trm4) further reinforces the idea of the existence of other enzymes with rather "relaxed" substrate specificity and thus the capability of

modifying numerous sites in cellular RNAs (tRNAs and probably in other RNA molecules). Further studies of the properties, functions, and substrate specificity of Trm4 should answer the question about the possible involvement of this enzyme in modification of other types of cellular RNAs.

MATERIALS AND METHODS

Chemicals, enzymes, and materials

[α^{32}P]-radiolabeled nucleoside triphosphates (400 Ci/mmol) and [^{3}H]AdoMet (15 Ci/mmol) were from Amersham (UK); nucleoside triphosphates, AdoMet, and *Aspergillus oryzae* RNAse T2 from Sigma (USA); bacteriophage T7 RNA polymerase, restriction enzymes, and IPTG from MBI Fermentas (Vilnius, Lithuania); Ni^{2+}-NTA-agarose from Qiagen (Germany); and anti His_6-tag antibodies (type G_{18}) from Santa Cruz Biotechnology, Inc. (USA). Synthetic oligodeoxynucleotides were purchased from MWG-Biotech (Germany) and used without further purification. Thin-layer cellulose plates were from Schleicher & Schuell (Germany) and all other chemicals from Merck Biochemicals (Germany).

Strains

E. coli strains JM103 (*supE thi-1 endA1 hsdR4 sbcB15 str*A Δ(*lac-proAB*) F′[*traD36 proAB*$^+$ *lacI lacZ*Δ*M15*]) and BL21(DE3) [hsdS gal (λcIts857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene1)] were used for cloning and expression of the His_6-tagged recombinant protein.

tRNA substrates

The plasmids carrying the synthetic genes of yeast tRNAs downstream of the T7 promoter are described elsewhere: $tRNA^{Asp}_{(GUC)}$ (Perret et al., 1990); $tRNA^{Phe}_{(GAA)}$ (Sampson et al., 1990); pre-$tRNA^{Phe}_{(GAA)}$ (Reyes & Abelson, 1989); $tRNA^{Ile}_{(UAU)}$ and $tRNA^{Tyr}_{(GUA)}$ (Szweykowska-Kulinska et al., 1994); and $tRNA^{Ser}_{(AGA)}$ (Himeno et al., 1997). They were kindly provided by R. Giegé and F. Fasiolo (Strasbourg, France), O.C. Uhlenbeck (Boulder, Colorado, USA), Z. Szweykowska-Kulinska (Poznan, Poland) and H. Himeno (Tokyo, Japan). Preparation of the plasmid carrying the sequence corresponding to the anticodon stem-loop of yeast $tRNA^{Phe}_{(GAA)}$ prolonged by a 19-nt-long natural intron (PheIVS minisubstrate) was described previously (Jiang et al., 1997). Likewise, the minisubstrate bearing the anticodon stem-loop prolonged by the 32-nt natural intron of yeast $tRNA^{Leu}_{(CUA)}$ downstream of the T7 promoter (LeuIVS minisubstrate) was prepared by PCR amplification of the corresponding sequence in the plasmid SUP53 CEN carrying the yeast *SUP53* precursor tRNA gene (Strobel & Abelson, 1986; kindly provided by C. Greer from Pasadena, California, USA). The PCR product was then cloned into the *Sma*I site of pUC118. Plasmid pDC952 bearing the minor *E. coli* $tRNA^{Arg}$ (gene *argU*) (Saxena & Walker, 1992) was kindly provided by Dr. J.R. Walker (Austin, Texas, USA).

In vitro T7 RNA-polymerase transcription using [α^{32}P]-radiolabeled nucleoside triphosphates and purification of the resulting T7 runoff tRNA transcripts by urea gels were performed as described elsewhere (Jiang et al., 1997).

Purification of total tRNA from *E. coli* and analysis of its nucleotide composition by postlabeling were performed as described previously (Constantinesco et al., 1998). Hydrolysis of tRNA fractions and the HPLC analysis of nucleosides by reverse-phase chromatography on Lichrosphere 100RP18 (C_{18}) 5 μm column (250 × 4.6 mm i.d.; Bischoff, Germany) were performed essentially as described in Gehrke & Kuo (1990).

Deoxyoligonucleotides and PCR amplification

The construct for expression of His_6-tagged recombinant Ybl024p in *E. coli* was prepared by the following procedure. The YBL024w ORF (2,055 bp) was amplified by PCR from total yeast genomic DNA using two primers that created a *Nhe*I restriction site at the ATG start codon (aaagctagcATG GCTAGAAGAAAGAATTTCAAAAAAGGG) and a *Bam*HI restriction site (aaaggatcCTCAATTAGCAGCGCTAGGAGC) immediately downstream of the TGA stop codon of the gene (the restriction sites in oligonucleotides are underlined). This manipulation allowed cloning of the ORF into pET28b vector (Novagen, USA) previously cut with *Nhe*I/*Bam*HI and created an in-frame fusion protein of six histidine residues joined by a spacer peptide of 13 amino acids (MGSSHHHHHHS SGLVPRGSHMAS) to the start methionine of ORF YBL024w. The resulting vector containing the fusion gene (pET28b/YBL024w) was transformed into *E. coli* BL21(DE3) cells.

Expression of Ybl024p in *E. coli* and purification of His_6-tagged recombinant protein

Expression in *E. coli* BL21(DE3) and purification of recombinant Ybl024p protein on Ni^{2+}-NTA-agarose column was performed essentially as described (Becker et al., 1997a). To improve the yield of recombinant Ybl024p upon expression in *E. coli,* the strain was cotransformed by the plasmid pDC952 bearing the gene *argU* encoding minor *E. coli* $tRNA^{Arg}_{(UCU)}$. Also the medium composition and time of IPTG-induction was optimized (see Results) to achieve a maximal yield of the recombinant protein. Protein electrophoresis in denaturing conditions was performed according to Laemmli (1970). Protein blotting and immunostaining were performed as described in Motorin et al. (1997).

In vitro enzymatic assay for tRNA:m^{5}C-methyltransferase activity

Activity of extracts and purified enzyme fractions was tested at 30 °C. The incubation mixture (50 μL) contained 100 mM Tris-HCl, pH 8.0, 100 mM ammonium acetate, 5 mM $MgCl_2$, 2 mM DTT, 0.1 mM ethylene diamine tetraacetic acid (EDTA), 20 μM AdoMet, 1–2 fmol of appropriately [^{32}P]-radiolabeled T7 transcripts as substrate, and 10–100 ng of purified Ybl024p (Trm4p) or cell extracts (*E. coli* or yeast) at a final concentration of 0.25 mg/mL. After incubation, the m^{5}C content in the radiolabeled transcripts was analyzed as described (Jiang et al., 1997). In brief, the RNA was extracted and completely hydrolyzed to 3′-nucleoside monophosphates by RNAse T2 ("nearest neighbor" analysis). Each hydrolysate was chromatographed on two-dimensional tlc plates and the radio-

activity in the spots was evaluated after exposure to a PhosphorImager screen (Molecular Dynamics, USA). Taking into account the total number of CMP residues in the tRNA molecule, the relative amount of m^{5}C formed in each transcript (expressed in mol per mol tRNA) can be determined. The accuracy of this method was found to be about ±0.05 mol m^{5}C per mol tRNA. Error bars shown on graphs represent confidence intervals calculated using Student's factor for probability 95%.

RNA-methyltransferase activity tests based on [^{3}H]-methyl group incorporation into nonradioactive transcripts were performed in similar experimental conditions, except that [^{3}H]AdoMet (25,000 cpm/pmol) was added to 1.3 μM final concentration and about 0.5 μg (20 pmol) of the transcript was used. The reaction was initiated by addition of the purified enzyme (200 ng). After incubation at 30 °C, the reaction was terminated by addition of cold 5% trichloracetic acid and the precipitate was collected by filtration through a GF/C filter (Whatman). The filter was washed with cold 5% trichloroacetic acid, dried, and the radioactivity was measured by liquid scintillation counting.

Preparation of disrupted ΔYBL024w yeast strain

Yeast disrupted strain bearing deleted ORF YBL024w was prepared by the one-step gene replacement approach (Wach et al., 1994). Gene of kanamycine resistance (provided by KanMX2 cassette) was amplified by PCR using two oligonucleotide primers complementary to KanMX2 cassette and to 42 nt 5′ upstream and 3′ downstream to YBL024w ORF respectively. Diploid yeast strain W303 was made competent by Li-acetate/PEG-4000 treatment and transformed by purified PCR product. Several G_{418}-resistant transformants were recovered and analyzed for correct integration by PCR amplification with specific oligonucleotides and restriction digestion. Haploid ΔYBL024w yeast strain was obtained by random spore analysis (Adams et al., 1997).

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