Purification of tRNA m^2G26-Methyltransferase from *Lupinus albus*

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ABSTRACT

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The enzyme, tRNA m^2G26-methyltransferase (TRM) catalyzes the transfer of methyl groups from S-adenosylmethionine to the 2-amino group of a guanosine residue at position 26 in tRNA. An activity assay to specifically measure the level of this enzyme in extracts from *Lupinus albus* was developed using tRNA isolated from yeast strains with and without N^2,N^2-dimethylguanosine-specific tRNA methyltransferase. This assay was used to monitor the purification of TRM from *Lupinus albus* (lupin) seeds using a combination of ammonium sulfate fractionation and diethylaminoethyl-Sepharose, hydroxyapatite, tRNA-affinity and adenosine-affinity chromatography. The most pure fractions showed a greater than 6000 - fold enrichment of TRM activity and showed only a few proteins at an apparent molecular weight of 66 kDa on a 10 % SDS-polyacrylamide gel, one of which cross-reacted with an antibody raised against the yeast *TRM1* protein. Taken together, these results suggest that this protein is the plant homolog of the yeast N^2,N^2-dimethylguanosine-specific tRNA methyltransferase enzyme.

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LIST OF ABBREVIATIONS

A	adenosine
ASP	aspartic acid
β-ME	β-mercaptoethanol
C	cytidine
CCA	cytidine-cytidine-adenosine
CNBr	cyanogen bromide
CPM	counts per minute
D	dihydrouridine
DEAE	diethylaminoethyl
DTT	dithiothreitol
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetracetic acid
G	guanosine
kDa	kilo daltons
mRNA	messenger ribonucleic acid
MWCO	molecular weight cut-off
PMSF	phenylmethylsulfonlyfluoride
RNase	ribonuclease
rRNA	ribosomal ribonucleic acid
SAH	S-adenosylhomocysteine
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate
T	thymidine
TLC	thin layer chromatography
TME	Tris-HCl β-mercaptoethanol
TRM	transfer ribonucleic acid m^2guanosine26-methyltransferase
tRNA	transfer ribonucleic acid
TΨC	thymidine-pseudouridine-cytidine
X.laevis	*Xenopus laevis*

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INTRODUCTION

A. TRANSFER RIBONUCLEIC ACIDS

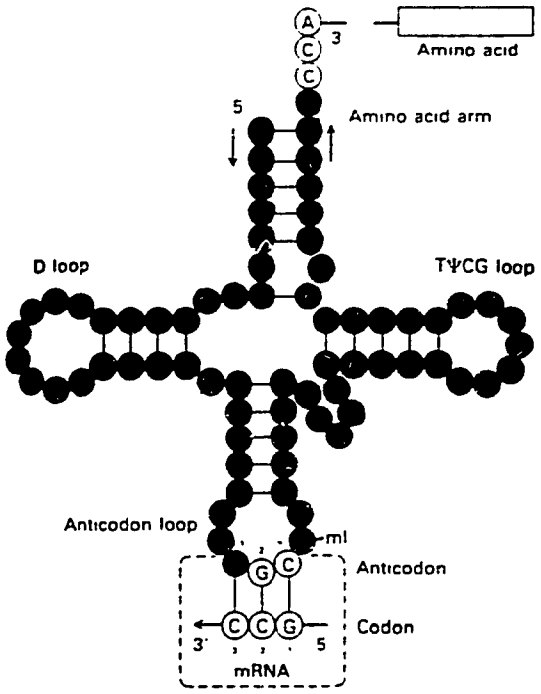
1. STRUCTURE

Transfer ribonucleic acids (tRNAs) are important macromolecules required for growth and maintenance of all cells. They are found in prokaryotes and eukaryotes and function mainly as amino acid carriers during protein synthesis. Transfer RNAs also function in cell wall biosynthesis (Zachau, 1978) and as primers for reverse transcriptase (Dahlberg, 1978).

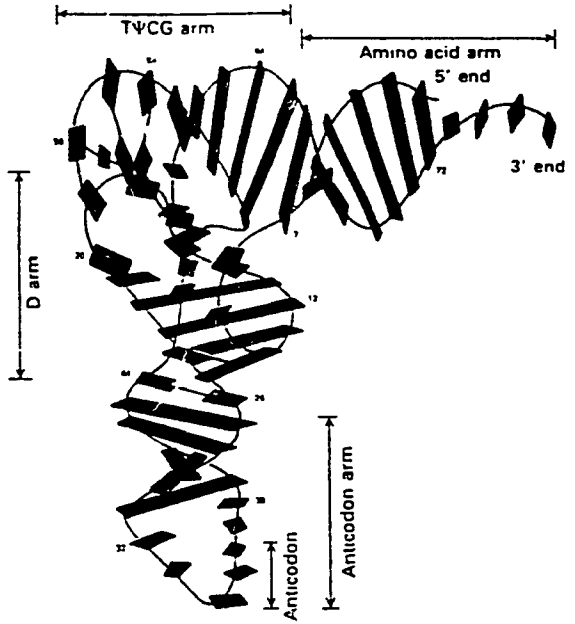
Transfer RNAs are single-stranded nucleic acid molecules composed of less than 100 nucleotides. All tRNAs have a 5' terminal phosphate, a 3'-terminal hydroxyl group and end with a cytidine, cytidine, adenosine (CCA) sequence at their 3' terminus (Figure 1). The primary sequence of tRNAs varies greatly but most share the common cloverleaf secondary structure first proposed by Holley *et al.* (1965). The cloverleaf secondary structure is made up of four stems, three of which have loops of variable lengths at their ends This secondary structure is stabilized by intrachain base pairing interactions which form the stems (Holley *et al.*, 1965). Amino acids are attached to the 3' end of the primary sequence at the end opposite to the anticodon loop in the folded tRNA (Figure 1) The other two loops also have unique characteristics, one of the loops usually contains the modified base, dihydrouridine (D) and the other loop generally has a thymidine, pseudouridine, cytidine (TΨC) sequence. Transfer RNAs also share a common L-shaped tertiary structure that is maintained by the stacking interactions

FIGURE 1: SECONDARY AND TERTIARY STRUCTURE OF TRANSFER RIBONUCLEIC ACIDS

(A)



(B)



The common cloverleaf secondary structure shared by most transfer ribonucleic acids with dihydrouridine, thymidine-pseudouridine-cytidine, anticodon and amino acid acceptor stems, 3' cytidine-cytidine-adenosine terminus and base pairing interactions (A). The common L-shaped tertiary structure shared by most transfer ribonucleic acids showing base stacking interactions that stabilize it (B) (Darnell *et. al.*, 1990).

between the bases and by H-bonding between specific invariant and semivariant bases (Figure 1). This common tertiary structure is required by tRNAs to bind to a common recognition site during protein synthesis. However, tRNAs require a diverse primary structure so that each is charged by a specific aminoacyl synthetase to ensure correct amino acids are added to the elongating polypeptide chain during protein synthesis.

2. BIOSYNTHESIS

a) Transcription

In *E.coli*, most tRNA genes are arranged in clusters. Of the 79 tRNA genes sequenced in *E.coli*, only 20 are single genes and the remaining genes are found in polycistronic operons which may contain other tRNAs, rRNAs or protein coding genes (Inokuchi and Yamao, 1995) The -35 and -10 elements, common to most *E.coli* genes, are the promoters used to initiate transcription of tRNA genes in *E.coli* (Inokuchi and Yamao, 1995). Termination is directed by a dyad symmetrical sequence and downstream thymidine residues (Inokuchi and Yamao, 1995).

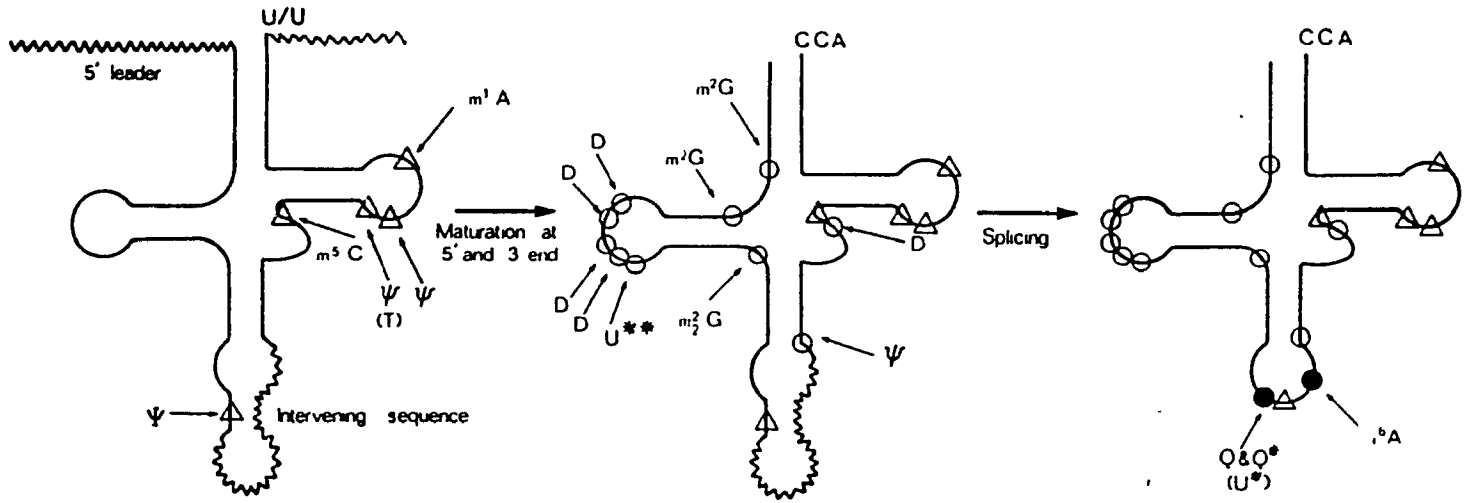
In eukaryotes, transcription of tRNA genes is under the control of two internal control regions that are within the tRNA gene sequence (Maréchal-Drouard *et al.*, 1993). These regions correspond to portions of the gene corresponding to the D and TΨC loops in the tRNA. Termination of transcription is thought to occur with poly T terminators (Maréchal-Drouard *et al.*, 1993).

b) Processing and CCA addition

The primary transcript of a tRNA gene undergoes many processing events to produce a mature, functional tRNA molecule These processing events include removal of 5' leader sequences, 3' trailer sequences and introns (if necessary). Other events that are required to produce a functional tRNA molecule include base modifications and the addition of cytidine, cytidine, adenosine residues to the 3' end of the tRNA (if necessary) (Figure 2).

In *E.coli*, if the tRNA primary transcript is part of a polycistronic precursor, endonucleases will separate the tRNA from the other portions of the primary transcript with specific cleavages. These cleavages are catalyzed by RNase III and RNase O which, however, do not yield the mature 5' and 3' termini. The endonuclease, RNase P, is responsible for the generation of the mature 5' terminus of all *E.coli* tRNAs. In fact, ribonuclease P serves this function not only in *E.coli* but also in all organisms studied to date (Deutscher, 1984). In *E.coli*, processing of tRNA precursors to yield a mature 3' terminus is catalyzed by exonucleases (Deutscher, 1984). These exonucleases include RNase II, RNase D, RNase BN, RNase T and RNase PH (Deutscher, 1984). RNase D will remove residues, one at a time from the exposed 3' end of the primary transcript until it reaches the CCA sequence At this point, there is a 30 - fold drop in the rate of hydrolysis by RNase D and the tRNA can be aminoacylated. RNase D dissociates from tRNA following every cleavage, and therefore, aminoacyl synthetases can compete for binding to tRNAs with a mature 3' end (Deutscher,

Figure 2: TRANSFER RNA MATURATION



The production of a mature transfer RNA molecule following transcription of a transfer RNA gene may involve: (1) processing of 5' leader and 3' trailer sequences (2) CCA addition (3) intron splicing and (4) nucleotide modification (Nishikura and Robertis, 1981).

1984). Aminoacylation will subsequently prevent further exonucleolytic cleavage The tertiary structure of tRNAs possibly also prevents further cleavage by RNase D or other exonucleases after the mature 3' end has been generated (Deutscher, 1984). RNase T catalyzes the removal of the terminal adenosine residue on the 3' end of mature tRNAs in a reaction known as "end turnover" (Deutscher, 1984) The tRNA is not further degraded and the adenosine residue can be replaced by tRNA nucleotidyltransferase yielding a functional tRNA.

The 3' terminus of all functional tRNA molecules contains a cytidine, cytidine, adenosine (CCA) sequence In *E.coli*, the 3' CCA is encoded in the tRNA genes and tRNA nucleotidyltransferase functions to repair tRNAs which have a damaged CCA sequence. In contrast, eukaryotic nuclear and organellar tRNA genes do not encode this sequence which must be added post-transcriptionally by tRNA nucleotidyltransferase (Deutscher, 1984).

In eukaryotes and archaebacteria, many tRNA genes contain introns (Deutscher, 1984) Most introns are located near the 3' end of the anticodon and contain a sequence that is complementary to the anticodon (Deutscher, 1984). Splicing enzymes cleave at the ends of the intron giving rise to two half tRNA molecules and releasing the intron. The two halves of the tRNA are then ligated to generate a mature tRNA molecule (Deutscher, 1984)

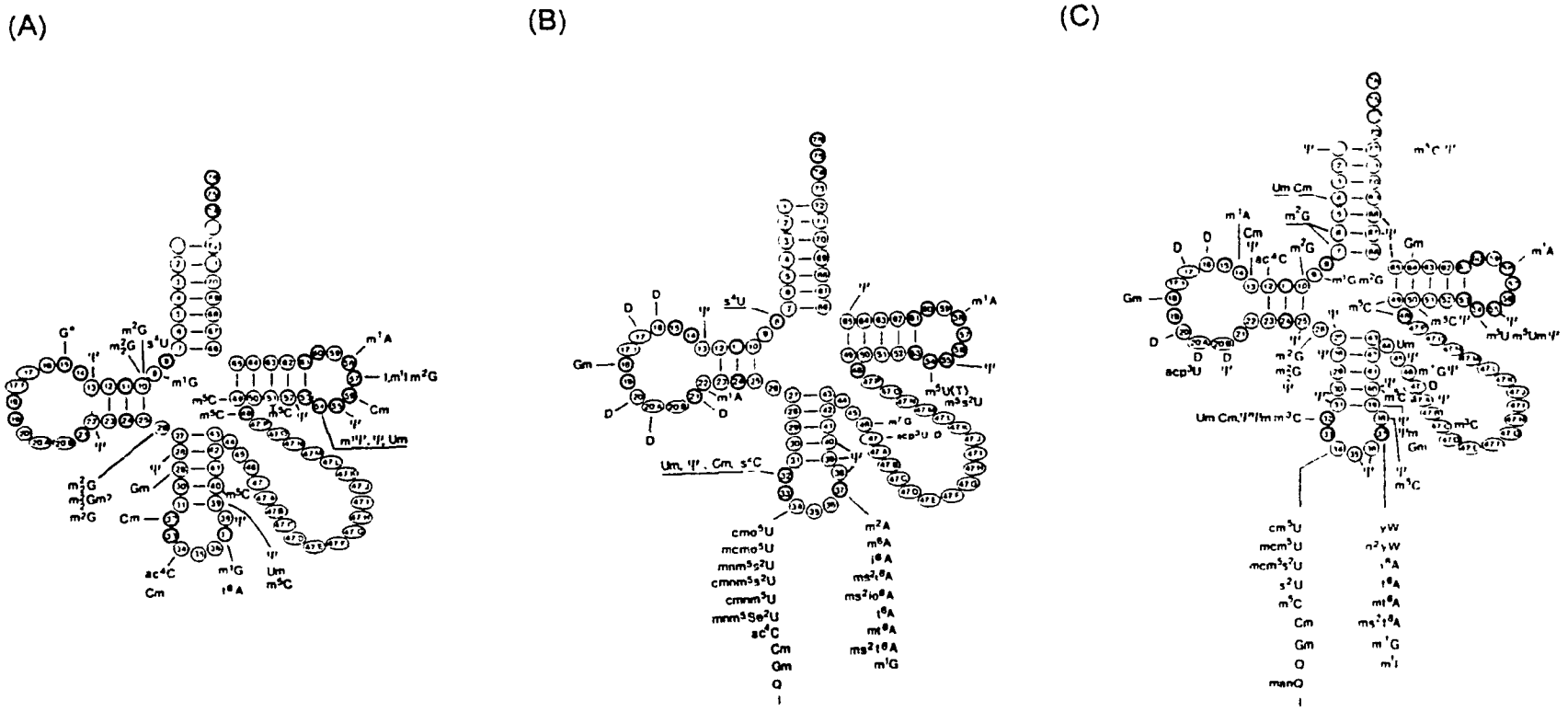
c) Base Modifications

Transfer RNA molecules contain not only the 4 standard nucleosides,

adenosine (A), cytidine (C), guanosine (G) and uridine (U) but also a large assortment of modified nucleosides. These modified nucleosides are found in prokaryotes and eukaryotes although the number and variety of modified nucleosides are greatest in eukaryotes where more than 80 modifications have been identified (Björk and Kohli, 1990) (Figure 3). The large variety of modifications identified to date includes ribosylations, isopentenylations, pseudouridylations and methylations (the most common) Some modified nucleotides such as dihydrouridine, pseudouridine, methyluridine, methylcytidine, 1-methylguanosine and inosine occur in all three kingdoms (Björk, 1995) whereas others are specific to a single kingdom (Nau, 1976)

The exact biological role(s) of tRNA modifications has(ve) not yet been elucidated due to lack of purified modifying enzymes, under-modified tRNAs or detectable phenotypes resulting from loss of a single modification. It has been suggested that some modifications affect the proper folding of the cloverleaf secondary structure of tRNA by decreasing the chances of incorrect base pair formation (Björk and Kohli, 1990) Other modifications may stabilize the tertiary structure by increasing or decreasing the H-bonding capability of specific bases (Björk and Kohli, 1990). It has also been suggested that the different modifications increase the diversity of the tRNA molecule over what is possible with only the four unmodified bases A,C,G,U and allow it to interact more

FIGURE 3: TRANSFER RNA MODIFICATION



Modifications identified in tRNA from archaebacteria (A), eubacteria (B) and eukaryotes (C) (Björk and Kohli, 1990). The greatest number of modifications can be seen in eukaryotic tRNA. The most common modified residues are 34 and 37. N^2,N^2-dimethylguanosine can be seen at position 26 in archaebacterial and eukaryotic tRNAs .

specifically with many different macromolecules such as proteins and nucleic acids (Persson, 1993) It is possible that different modifications have different impacts on tRNA stability in the cell or on how these tRNAs are involved in regulation, interaction and recognition during different cellular processes (Björk and Kohli, 1990).

Although modification of tRNAs occurs post-transcriptionally and previously modified mononucleosides are not added during transcription, the chronological order of tRNA modifications has not been fully determined. Modification can continue throughout the tRNA maturation process with some modification occurring prior to processing (Nishikura and Robertis, 1981, Melton *et al.*, 1980) while others occur after processing (Nishikura and Robertis, 1981, Melton *et al.*, 1980).

Residues 34 (Wobble position) and 37 (next to the anticodon) contain the largest variety of modifications. The modifications at these two positions may increase the specificity, the fidelity and the efficiency of the tRNA molecule during protein synthesis (Yokoyama and Nishimura, 1995). The modifications at position 34 allow the codon:anticodon degenerate interactions proposed by the Wobble Hypothesis (Crick, 1966) by affecting either the base pairing between the codon and the anticodon or the conformational rigidity of the base (Persson, 1993). The modifications at position 37 also may help strengthen the interaction of the anticodon with the codon (Yokoyama and Nishimura, 1995)

Nucleoside modification is catalyzed by tRNA modifying enzymes. These

modifications can occur in the ribose ring or in the purine or pyrimidine base of the nucleotide. Transfer RNA modification enzymes are very specific for tRNAs, and do not catalyze modification of DNA, mononucleosides, synthetic polynucleotides, rRNA or mRNA (Kerr and Borek, 1973, Rodeh *et al.*, 1967). The large number of modifications found in eukaryotic tRNAs suggests that a large number of modifying enzymes is required to catalyze all of these reactions.

Enzymes responsible for tRNA modifications may be specific for a specific nucleoside, a specific position within the nucleoside and a specific position within the tRNA molecule that they modify. Enzymes may recognize primary, secondary or tertiary structure of the tRNA molecule or more specifically a pattern of bases in the tRNA molecule, or even a combination of both structure and base pattern (Björk and Kohli, 1990, Shershneva *et al.*, 1971). The enzymes responsible for tRNA modifications have proven difficult to purify because they are present in the cell in low amounts (Björk and Kohli, 1990). Because my research has concentrated on the purification of a specific tRNA methylase, tRNA m^2G26-methyltransferase, I will now concentrate on what is known about tRNA-specific methyltransferase enzymes. The identification of tRNA modification enzymes was first accomplished using *E.coli* extracts. Methionine starved *E.coli* cells lacked methylations in tRNA. The isolated tRNAs were subsequently methylated when crude extracts of non-methionine starved *E.coli* cells were used (Fleissner and Borek, 1962).

B. N^2,N^2-DIMETHYLGUANOSINE-SPECIFIC tRNA METHYLTRANSFERASE

1. BACKGROUND

The methyl donor in methyltransferase-catalyzed reactions is S-adenosylmethionine (SAM) in all organisms except for gram positive bacteria which use N^5,N^{10}-methylenetetrahydrofolate (Björk and Kohli, 1990) Most methyltransferases are inhibited by the end product of the methyltransfer reaction, S-adenosylhomocysteine (SAH). Transfer RNA methyltransferases are specific for transferring methyl groups from S-adenosylmethionine to tRNA.

Efforts have been made to purify tRNA methyltransferases from many sources (Kuchino and Nishimura, 1970, Glick *et al.*, 1978, Salas and Dirheimer, 1979, Greenberg and Dudock, 1980, Keith *et al.*, 1980, Turkington and Riddle, 1970, Pope and Reeves, 1978, Greenberg and Dudock, 1979, Kerr, 1979, Izzo and Gantt, 1977, Rjalmarsson *et al.*, 1983, Svensson *et al.*, 1969, Björk and Svensson, 1969, Liau *et al.*, 1972), so that the effects of each tRNA methylation can be studied separately. However, only a few tRNA methyltransferases have been characterized to date because of (1) their instability during both short term storage at 0 - 4° C and long term storage at -70°C (Wierzbicka *et al.*, 1975, Agris *et al.*, 1974, Glick and Leboy, 1977, Chan and Fraser, 1972), (2) their low abundance during cell fractionation (Björk and Kohli, 1990, Rjalmarsson *et al.*, 1983) and (3) the lack of suitable under-methylated tRNA substrates (Chan and Fraser, 1972).

2. CHARACTERIZATION OF N^2,N^2-DIMETHYLGUANOSINE-SPECIFIC tRNA METHYLTRANSFERASE

N^2,N^2-dimethylguanosine-specific tRNA methyltransferase has been characterized in wheat embryos (Kwong, 1975), *Tetrahymena pyriformis* (Reinhart *et al.*, 1986) and HeLa cells (Agris *et al.*, 1974). This enzyme catalyzes the dimethylation of the 2-amino group at G_{26} in tRNAs (see Figure 3).

N^2,N^2-dimethylguanosine-specific tRNA methyltransferase from wheat embryos was partially fractionated using DEAE-cellulose chromatography and assayed using *E.coli* tRNA (Kwong, 1973). However, attempts to obtain a pure protein were unsuccessful. This enzyme was characterized as N^2,N^2-dimethylguanosine-specific tRNA methyltransferase because N^2,N^2-dimethylguanosine-containing tRNAs were only isolated when tRNAs undermethylated at the appropriate guanosine residue were used as substrate.

There is only one report of N^2,N^2-dimethylguanosine methyltransferase being purified to homogeneity (Reinhart *et al.*, 1986). The *Tetrahymena pyriformis* enzyme was enriched 845 - fold using DEAE-cellulose, phosphocellulose and Blue A affinity chromatography and the pure enzyme was able to catalyze the mono- and dimethylation at G_{26} of *E.coli* tRNA and tRNA isolated from the yeast *trm*1 mutant. Its purity was defined by a single band on silver staining of an SDS-polyacrylamide gel.

Recent studies using crude enzyme preparations are helping to identify the methylating identity elements of *Xenopus laevis* (Edqvist *et al.*, 1992) and *Saccharomyces cerevisiae* (Edqvist *et al.*, 1994) tRNAs required for N^2,N^2-dimethylguanosine specific tRNA methyltransferase activity Yeast $tRNA^{Asp}$ is a good substrate to use to study these identity elements because it normally lacks methyl groups on the 2-amino group at G_{26} Using synthetic variants that contain two altered dihydrouridine stem base pairs (C11:G24 and G10:C25 instead of U11:A24 and A10:U25 as with the wild type tRNA) dimethylation of G_{26} was achieved in *X. laevis* extracts (Edqvist *et al.*, 1992) The introduction of these base pairs altered the microenvironment of G_{26} such that its 2-amino group was more solvent exposed, and capable of being dimethylated by N^2,N^2-dimethylguanosine specific tRNA methyltransferase (Edqvist *et al.*, 1992). Based on these studies a two step kinetic mechanism for G_{26} dimethylation was proposed because monomethylation occurred at a faster rate than dimethylation (Edqvist *et al.*, 1992). The kinetics of the methylation reaction in *Tetrahymena pyriformis* also showed that there was a lag time between mono- and dimethylation (Reinhart *et. al.*, 1986). There are at least three possible explanations for these kinetic data: (1) the two methylation reactions need different physiological conditions, (2) the enzyme may require dissociation from the tRNA molecule between the mono and dimethylation so that it can release the S-adenosylhomocysteine product molecule and pick up an S-adenosylmethionine substrate molecule or change conformations to catalyze

dimethylation or (3) there are two enzymes, each catalyzing a distinct methylation (Edqvist *et al.*, 1992, Reinhart *et al.*, 1986).

Evidence to support the third hypothesis (that there are two distinct methylation enzymes in higher eukaryotes) comes from the observation that the mutated yeast $tRNA^{Asp}$ which is not methylated using yeast extracts is monomethylated using *X.laevis* (Edqvist *et al.*, 1992), rat (Pegg, 1974) and mouse (Pegg, 1974) extracts. It is possible that identity elements differ between yeast and higher eukaryotes for the monomethylation reaction or that another enzyme exists with different identity elements that monomethylates yeast $tRNA^{Asp}$ at position G_{26} in higher eukaryotes (Edqvist *et al.*, 1992).

Dimethylation of yeast $tRNA^{Asp}$ by yeast N^2,N^2-dimethylguanosine-specific tRNA methyltransferase was only accomplished after an additional base also was inserted at position 47 in the variable loop along with the two dihydrouridine stem base pairs, C11:G24 and G10·C25 (Edqvist *et al.*, 1994). Thus, the methylating enzymes of yeast and *X.laevis* do not have the same identity elements because an extra base was required in the variable loop by the yeast enzyme that was not required by the *X. laevis* enzyme (Edqvist *et al.*, 1994). The introduction of the two dihydrouridine stem base pairs and a base in the variable loop caused yeast $tRNA^{Asp}$ to be mischarged (Edqvist *et al.*, 1992). In this case, apparently having a dimethylated G_{26} was not as biologically important as being aminoacylated (Edqvist *et al.*, 1992).

3. GENETIC ANALYSIS

To date, only a single N^2,N^2-dimethylguanosine-specific tRNA methyltransferase has had its coding region sequenced Complementation was used to isolate the TRM1 (transfer RNA modification) gene encoding this enzyme in yeast (Ellis *et al.*, 1986). The enzymatic activity encoded by the open reading frame was able to mono- and dimethylate *E.coli* tRNAs *in vivo* and tRNAs isolated from the *trm*1 yeast strain, SN1015-2a *in vitro* (Ellis *et al.*, 1986). Both *E.coli* and SN1015-2a tRNA lack methylation at G_{26} (Reinhart *et al.*, 1986, Phillips and Kjellin-Straby, 1967).

Two enzymes that differ in length by 16 amino acids at the amino-terminus are the result of translation of two different sized messages encoded by the TRM1 gene (Ellis *et al.*, 1987). While the enzyme containing the 16 additional amino terminal amino acids is confined to the mitochondrion, the protein lacking these 16 amino acids functions both in the nucleocytoplasm and the mitochondrion. However, both forms of the enzyme contain mitochondrial (Ellis *et al.*, 1987) and nuclear targeting signals (Li *et al.*, 1989).

Indirect immunofluorescence studies showed that amino acids 70 - 213 from N^2,N^2-dimethylguanosine-specific tRNA methyltransferase were able to localize a fusion protein to the nucleus (Li *et al.*, 1989) indicating that a nuclear localization signal resides between amino acids 70 and 213 in N^2,N^2-dimethylguanosine-specific tRNA methyltransferase. Amino acids 95 - 102 were identified in further studies to define the nuclear localization signal of N^2,N^2-

dimethlguanosine-specific tRNA methyltransferase that were similar to other known nuclear localization signals (Rose *et al.*, 1992). Exchanging 5 lysines with 5 glutamic acids within residues 95 - 102 abolished nuclear localization. The introduction of residues 95 - 102 into β-galactosidase caused its localization to the nucleus in yeast cells. Therefore, residues 95 - 102 in N^2,N^2-dimethylguanosine-specific tRNA methyltransferase were both necessary and sufficient for nuclear targeting suggesting that this sequence defines the nuclear localization signal of N^2,N^2-dimethylguanosine-specific tRNA methyltransferase (Rose *et al.*, 1992). Likewise, similar studies suggest that the first 48 amino acids of N^2,N^2-dimethylguanosine-specific tRNA methyltransferase contain the mitochondrial targeting signal of N^2,N^2-dimethylguanosine-specific tRNA methyltransferase (Ellis *et al.*, 1989, Li *et al.*, 1989 and Rose *et al.* 1992).

4. THIS PROJECT

The correct intracellular localization of proteins is dependent on their targeting signals. N^2,N^2-dimethylguanosine-specific tRNA methyltransferase in yeast contains both nuclear and mitochondrial targeting signals. Therefore, it is of interest to study this protein in plants to see if one gene with multiple targeting signals is responsible for the enzymatic activities in the nucleocytoplasm, the mitochondrion and the chloroplast or if there are multiple genes each coding for the activity present in one compartment Also little is known about enzymes responsible for methylating tRNAs compared to DNA methylases and other RNA

methylases. Therefore, purification of this protein will allow us to identify active site residues essential for structure and function, as well as SAM and tRNA binding sites.

MATERIALS AND METHODS

A. PREPARATION OF CRUDE EXTRACT

One kilogram of dry *Lupinus albus* seeds was ground manually in 4 l of 50 mM sodium acetate buffer (pH 6) (Cudny *et al.*, 1978), using a meat grinder with a grating of 4 mm (Shanmugam, 1994). Seeds and buffer were continuously added to the meat grinder and the slurry was collected in a plastic container. The total slurry was divided into 12 x 500 ml Nalgene centrifuge bottles and centrifuged at 7000 rpm for 30 minutes at 4°C in a J2-HS centrifuge (Beckman) using the JA-10 rotor to pellet the large seed debris. The resulting supernatant was filtered through one layer of Miracloth (Calbiochem).

B. AMMONIUM SULFATE FRACTIONATION

Ammonium sulfate that had been ground to a fine powder using a mortar and pestle was added slowly over a period of 10 - 15 minutes to the filtrate with stirring to a final concentration of 30% saturation (176 g/l at 4°C). After the ammonium sulfate was dissolved, the filtrate-ammonium sulfate mixture was left undisturbed at 4°C for 30 minutes and then transferred to 6 x 500 ml Nalgene centrifuge bottles. These were centrifuged in the J2-HS centrifuge using the JA-10 rotor at 4°C for 30 minutes at 10 krpm. The 0 - 30% pellets were resuspended and combined in a total of 200 ml TME buffer (50 mM Tris-HCl [pH 8] and 1 mM β-mercaptoethanol) and stored at 4°C until the ammonium sulfate

fractionation was complete. Additional powdered ammonium sulfate was added slowly to the supernatant, over a period of 20 - 30 minutes, with stirring to bring it to 50% saturation (an additional 126 g/l). After the ammonium sulfate was dissolved, the mixture was left undisturbed at 4°C for 30 min, transferred to 6 x 500 ml Nalgene centrifuge bottles and centrifuged at 4°C in the J2-HS centrifuge using the JA-10 rotor at 10 krpm for 30 minutes. The 30 - 50% pellets were resuspended and combined in a total of 100 mls of TME buffer and stored at 4°C.

C. DIALYSIS

The 0 - 30% and 30 - 50% ammonium sulfate fractions and an aliquot of the 50% supernatant were dialyzed in Spectra/Por 1 dialysis tubing (molecular weight cut off of 6-8 kDa) against 6 l of TME buffer overnight at 4°C. Dialysis was repeated with 6 l of fresh TME buffer for a minimum of 4 hours.

D. PROTEIN CONCENTRATION

Protein concentrations were determined following the microassay instructions supplied by BioRad. This assay is based on the Bradford dye-binding procedure (Bradford, 1976). The dye reagent concentrate was diluted 1:4 with distilled water and 800 µl of the diluted dye reagent concentrate used with an aliquot of the protein sample and water to give a final volume of 1000 µl. The samples were mixed in a 1.5 ml cuvet and incubated at room temperature

for a minimum of five minutes. Absorbances were measured at 595 nm on a Novaspec II Visible Spectrophotometer (Pharmacia).

A new standard curve was made from known amounts of bovine serum albumin (0, 2, 4, 6, 8 and 10 µg) each time fresh Bio-Rad dye reagent was used. Linear regression analysis was used to determine the protein concentration of different protein samples from the standard curve.

E. ISOLATION OF YEAST TRANSFER RNA

The yeast strains, W303-1b (*MAT*a, *ade*2-1, *his*3-11/15, *leu*2-3/112, *ura*3-1, *trp*1-1, *can*1-100)) and SN1015-2a (*MAT*α, *SUP*4, *trm*1, *trm*2, *gal*1, *gal*7, *ade*2-1, *leu*1, *ura*3, *met*, *lys*2-1, *trp*, *ura*3-1) were used as sources of tRNA. The tRNA isolation procedure was adapted from Hopper and Schultz (1980). Briefly, yeast cells were grown in YPD medium (2% peptone, 1% yeast extract, 2% dextrose) for 16 - 20 hours at 30°C with shaking. The cells were harvested when the OD_{600nm} was greater than 1. The cells were pelleted by centrifugation at 5000 rpm using the JA-10 rotor in the J2-HS centrifuge for 10 minutes at 4°C. The pelleted cells were resuspended in 3 ml TSE buffer (0.01 M Tris-HCl [pH 8], 0.1 M NaCl and 0.01 M EDTA [pH 8]) per 100 ml of YPD medium and 3 ml of Tris-equilibrated phenol were added. This mixture was incubated at 36°C for 60 minutes with shaking and then placed at 4°C for at least 120 minutes. The mixture was transferred to 50 ml Oak Ridge tubes (Nalgene) and centrifuged at 15 krpm using the JA-20 rotor in the J2-HS centrifuge for 20 minutes at 4°C to

separate the two phases. The aqueous phase was transferred to another Oak Ridge tube and an equal volume of isopropanol was added. The Oak Ridge tube was stored at -20°C overnight and centrifuged at 15 krpm using the JA-20 rotor in the J2-HS centrifuge for 30 minutes at 4°C to pellet the tRNAs. The resulting pellet was washed with 80% ethanol and dried in a desiccator under vacuum. The pellet was resuspended in 10 ml dH_20 and extracted two times with phenol, four times with phenol-chloroform (1:1) and two times with chloroform. To the aqueous phase was added 1/10th volume 3 M sodium acetate and an equal volume of isopropanol. This was stored at -20°C overnight and centrifuged at 15 krpm using the JA-20 rotor in the J2-HS centrifuge for 30 minutes at 4°C. The resulting pellet was washed with 80% ethanol and dried in a desiccator under vacuum The pellet was resuspended in 1 ml of dH_20 and the concentration was determined by measuring the absorbance at 260 nm using the Lambda 3 Spectrophotometer (Perkin-Elmer).

F. TRANSFER RNA m^2G26-METHYLTRANSFERASE ACTIVITY

The procedure of Ellis *et al.* (1986) with several modifications was used to measure tRNA m^2G26-methyltransferase (TRM) activity. In each activity assay, 5 x TRM cocktail consisted of 100 µg of tRNA, 0.48 µCi [^{3}H]-SAM (15 Ci/mmol), 0.72 µg SAM, 0.5 M Tris-HCl (pH 8), 0.5 mM EDTA (pH 8), 50 mM $MgCl_2$, 0.5 M NH_4Cl and 5 mM DTT in a volume of 30 µl. The amount of protein that was

added to each activity assay varied depending on the concentration of the protein sample but was never more than 120 µl (or 500 µg). If the amount of protein was contained in a volume of less than 120 µl, dH_20 was added to bring the total volume to 150 µl. The reaction mixtures were incubated in microfuge tubes in a 37°C water bath for 60 minutes. The activity assay was stopped by adding the reaction mixtures (after centrifugation in an Eppendorf microfuge for 10 seconds at 13 krpm) to 850 µl of ice-cold dH_20 in test tubes and immediately adding 1 ml of ice-cold 2 N HCl. This mixture was left on ice for at least 10 minutes and the precipitated material was collected by filtration through 1 N HCl pre-soaked GF/C glass fibre filters (Whatman) using a sampling manifold (Millipore). Each filter was washed with 3 x 10 ml of ice-cold 1 N HCl and 5 ml of ice-cold 99% ethanol. The filters were dried at 150°C for 5 minutes and placed in scintillation vials containing 5 ml Cytoscint scintillation fluid (ICN). The counts were measured in an LKB WALLAC - 1218 RACKBETA scintillation counter.

G. COLUMN CHROMATOGRAPHY

All chromatographic procedures were done in a 4°C cold room using a P-1 peristaltic pump (Pharmacia) and a Redi-Frac fraction collector (Pharmacia).

1. DEAE CHROMATOGRAPHY

A 2.5 x 30 cm Econo-Column (Bio-Rad) was packed with DEAE Sepharose Fast-Flow (Pharmacia) resin at a flow rate of 9 ml/min and

equilibrated with TME buffer overnight at a flow rate of 6 ml/min. The total bed volume was 140 mls. The 30 - 50% dialyzed fraction (approximately 150 - 180 ml) was centrifuged at 20 krpm using the JA-20 rotor at 4°C for 60 minutes in a J2-HS centrifuge. This fraction was diluted 3-fold with TME buffer and loaded onto the column at a flow rate of 6 ml/min. The column was washed with a minimum of 5 l of TME buffer overnight at a flow rate of 6 ml/min so that the amount of protein collected in the last fractions was below the level of detection with the Bradford assay ($OD_{595nm} < 0.1$). The adsorbed protein then was eluted with 800 mls of a 0-200 mM NaCl linear gradient at a flow rate of 8 ml/min. The linear gradient was formed in a gradient maker (Bethesda Research Laboratories). After the gradient was finished, the column was washed with an additional 50 mls of 200 mM NaCl. The column was stripped of remaining proteins with 500 mls of 2 M NaCl, washed overnight with 6 l of dH_20 and equilibrated again with 500 mls of TME buffer prior to reuse.

The fraction sizes were 12 ml and every third or fifth fraction was assayed for protein and TRM activity. The active fractions were pooled and loaded directly onto a hydroxyapatite column.

2. HYDROXYAPATITE CHROMATOGRAPHY

A 2.5 x 20 cm Econo-Column (Bio-Rad) was packed with hydroxyapatite (Bio-Rad) resin at a flow rate of 1.5 ml/min and equilibrated with TME buffer overnight at a flow rate of 1 ml/min. The total bed volume was approximately 60

mls. The pooled, active, bound DEAE fractions (140 - 160 mls) were loaded onto this column at a flow rate of 1 ml/min. The column was washed with a minimum of 250 mls of TME buffer until the amount of protein in the collected fractions was below the level of detection with the Bradford assay (OD_{595nm} < 0.1). The adsorbed proteins were eluted with 500 mls of a 10 - 500 mM sodium phosphate (pH 6.5) linear gradient at a flow rate of 1 ml/min and 8 ml fractions were collected. Elution was continued with an additional column volume of 500 mM sodium phosphate buffer (pH 6.5) and the resin immediately washed with 5 column volumes of dH_20 to remove the 500 mM sodium phosphate buffer. The column was then equilibrated with 2 l of TME buffer overnight prior to reuse.

Every third or fifth fraction was assayed for protein and TRM activity. The active fractions were pooled and dialyzed for 5 hours against 20 mM sodium phosphate buffer (pH 6) in Spectra/Por 1 dialysis tubing (MWCO 6 - 8 kDa) in preparation for tRNA-Sepharose affinity chromatography.

3. tRNA-SEPHAROSE AFFINITY CHROMATOGRAPHY

The tRNA affinity resin was made using the following procedure. Five grams of CNBr-activated Sepharose 4B freeze dried powder (Pharmacia) were resuspended in 15 mls of 1 mM HCl. The swollen gel was washed for 15 minutes with 1 l of 1 mM HCl on a sintered glass funnel. The ligand was total wheat tRNA type V (Sigma). Lyophylized tRNA (75 mg) was dissolved in 25 ml coupling buffer (0.1 M sodium bicarbonate [pH 8.3] and 0.5 M NaCl) and mixed

with the gel in a 50 ml polypropylene tube by end-over-end rotation at 10 rpm at 4°C overnight. Uncoupled ligand was removed from the gel by washing with 100 volumes of coupling buffer in a sintered glass funnel. The remaining active groups were blocked with 0.1 M Tris-HCl buffer (pH 8) with end-over-end rotation at 10 rpm overnight at 4°C. The gel was washed with three alternating cycles of 0.1 M acetate (pH 4) with 0.5 M NaCl and 0.1 M Tris-HCl (pH 8) with 0.5 M NaCl.

The resin was packed in a 1.5 x 20 cm Econo-Column (Bio-Rad) at a flow rate of 1.5 ml/min. The total bed volume was 33 ml. The resin was washed with dH_20 and equilibrated in 20 mM sodium phosphate (pH 6). The dialyzed fractions from the hydroxyapatite column were loaded onto the tRNA affinity column at a flow rate of 1 ml/min. The column was washed with 20 mM sodium phosphate (pH 6) buffer containing 1 mM ß-mercaptoethanol until there was no detectable protein with the Bradford assay ($OD_{595nm} < 0.1$) in the 13 ml fractions. Washing was continued with 50 mM Tris-HCl (pH 8.5) containing 1 mM ß-mercaptoethanol until there was no detectable protein with the Bradford assay ($OD_{595nm} < 0.1$) in the 13 ml fractions. The remaining absorbed proteins were eluted with 50 mM Tris-HCl (pH 8.5) containing 250 mM NaCl and 1mM ß-mercaptoethanol at a flow rate of 1 ml/min until there was no detectable protein with the Bradford assay ($OD_{595nm} < 0.1$) in the 2 ml fractions. At the end of each run, the column was stripped with 2 M NaCl, washed with dH_20 and equilibrated with 20 mM sodium phosphate (pH 6) buffer.

Each of the eluted fractions was assayed for protein. The fractions found to contain protein were then assayed for TRM activity. The active fractions were pooled and frozen at -86°C in 10 - 15% glycerol.

4. ADENOSINE-AFFINITY CHROMATOGRAPHY

This resin was prepared in the following manner by Dr. Franck James (University of Florida). Adenosine-mono-phosphate-agarose (Sigma) was washed with distilled water in a glass sintered funnel and then treated with alkaline phosphatase (1000 u)(Sigma) overnight at room temperature with gentle end-over-end rotation. The resin was washed with water to remove the hydrolyzed phosphates. This resin, with a bed volume of 400 µl, was packed in a 10 ml Econo-column (Bio-Rad) and equilibrated with TME buffer.

The active, pooled tRNA affinity fractions were dialyzed against TME buffer in Spectra/Por 1 dialysis tubing (MWCO 6 - 8 kDa) for 4 hours or alternately diluted 10-fold and loaded directly onto the adenosine-affinity column at a flow rate of ≈1 ml/min. The column was washed with 30 mls of TME buffer and with 200 mM NaCl with 1 mM ß-mercaptoethanol. Finally the column was washed with 200 mM NaCl containing 1 mM S-adenosylmethionine (SAM) and 1 mM ß-mercaptoethanol. Fractions (1 ml) were collected and most assayed for protein and TRM activity. The fractions containing 1 mM SAM had to be dialyzed in Spectro/Por 1 dialysis tubing (MWCO 6 - 8 kDa) for a minimum of 4 hours against TME buffer to reduce the concentration of unlabelled SAM prior to

the TRM activity assay.

H. CONCENTRATION OF FRACTIONS

Fractions eluted with 200 mM NaCl with 1 mM SAM from the adenosine-affinity column (total volume of each step was approximately 30 mls) were transferred to Spectra/Por 1 dialysis tubing (MWCO of 12 - 14 kDa) at 4°C and the tubing was overlaid with polyethylene glycol flakes (Carbowax PEG 20 000, Fisher). The wet PEG flakes were changed every hour until the volume had decreased to less than one milliliter.

Other fractions eluted from the adenosine-agarose affinity column were dialyzed in Spectra/Por 1 dialysis tubing (MWCO of 12 - 14 kDa) in 1 mM Tris-HCl (pH 8) and then lyophillized in a Speed-Vac (Savant). The fractions were suspended in 50 µl dH_2O and used in SDS-polyacrylamide gel electrophoresis.

I. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Mini SDS-polyacrylamide gels were made and electrophoresed using the Mini Protean II Electrophoresis System (Bio-Rad). Separating gels and stacking gels were made according to the procedure provided with the apparatus Briefly, separating gels contained either 7.5%, 10% or 12% acrylamide/N,N'-methylenebisacrylamide (37.5:1 ratio), 0.375 M Tris-HCl (pH 8.8), 0.1% SDS, 0.05% TEMED (N,N,N',N'-tetramethylethylenediamine) and 0.05% ammonium persulfate. Stacking gels contained 4% acrylamide/N,N'-

methylenebisacrylamide (37.5:1 ratio), 0.125 M Tris-HCl (pH 6.8), 0.1% SDS, 0 05% N,N,N',N'-tetramethylethylenediamine (TEMED) and 0.05% ammonium persulphate The 5 x protein loading (sample) buffer contained 0.0625 M Tris-HCl (pH 6 8), 25% glycerol, 2% SDS, 0.72 M β-mercaptoethanol and 0.05% bromophenol blue. Samples were diluted 1:4 with 5 x protein loading buffer and boiled for 5 minutes prior to loading them onto the gel Low-range molecular weight markers (Bio-Rad) were also run on the gels in order to estimate the sizes of the proteins Gels were electrophoresed until the bromophenol blue dye had reached the bottom of the gel.

J. GEL STAINING

In order to visualize the proteins after electrophoresis, the gels were stained with the Bio-Rad Silver Stain Plus kit After electrophoresis, the gels were soaked in fixing solution (10% v/v Bio-Rad Fixative Enhancer Solution, 50% v/v methanol and 10% v/v acetic acid) for 20 minutes. The gels then were rinsed twice with distilled water for 20 minutes and placed in developing solution (containing 2.6% w/v Development Accelerator Reagent, 5% v/v Silver Complex Solution, 5% v/v Image Development Reagent and 5% v/v Reduction Moderator Solution) until satisfactory band intensities were observed. The developing process was stopped with stop solution (5% acetic acid).

K. GEL DRYING

The gels and cellophane membrane paper were presoaked in 3% glycerol and dried under vacuum using a Model 583 Gel Dryer (Bio-Rad) according to the instructions provided by the manufacturer.

L. GEL TRANSFER

After electrophoresis, the gel that was to be transferred, as well as filter pads (Bio-Rad), 3mm paper (Canada-Wide Scientific) and nitrocellulose membrane (Millipore) were soaked in blotting buffer (25 mM Tris, 192 mM glycine and 20% methanol [JT Baker]) for 30 minutes. The gel was blotted onto nitrocellulose membrane (Whatman) overnight (16 hours) at ≈130 mA at 4°C using the Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according to the instructions provided.

M. WESTERN BLOT

Western blots were done using the Immun-Blot Assay Kit (Bio-Rad). The nitrocellulose membrane was removed from the Trans-Blot Apparatus and was soaked in TBS buffer (20 mM Tris-HCl [pH 7.5] and 0.5 M NaCl) for 30 minutes. The nitrocellulose membrane was blocked for 2 hours with 3% gelatin in TTBS buffer (20 mM Tris-HCl [pH 7.5], 0.5 M NaCl and 0.05% Tween-20) The membrane was washed for 30 minutes in TBS buffer and then for 30 minutes in TTBS buffer. The yeast *TRM1* primary antibody (kindly provided by Dr Nancy

Martin, University of Louisville) was diluted in 1% gelatin in TTBS buffer (1:500) and incubated with the nitrocellulose membrane overnight at room temperature in a Seal-a-Meal bag with gentle shaking. After removing the primary antibody, the membrane was washed with TTBS buffer twice for 30 minutes each time. The alkaline phosphatase conjugated goat-anti-rabbit secondary antibody (Bio-Rad) was diluted (as per instructions supplied by Bio-Rad) in 1% gelatin in TTBS buffer (1:3000) and incubated with the membrane for 2 hours at room temperature The secondary antibody was removed and the membrane was washed twice with TTBS buffer for 15 minutes each time and twice with TBS buffer for 5 minutes each time. The color development solution (prepared by mixing 100 µl of AP color reagent A and 100 µl of AP color reagent B in 10 ml of AP color development buffer) was incubated with the membrane at room temperature until the intensity of the bands was satisfactory. The color developing reaction was stopped by placing the membrane in distilled water for 10 minutes. The membrane was then stored permanently in distilled water.

N. PREPARATION OF CRUDE YEAST PROTEIN

Yeast cells were inoculated into 5 ml of YPD media and grown for 16 - 20 hours (to an OD_{600nm} greater than 1) at 30°C with shaking. The cells were pelleted by centrifugation at 5000 rpm using the JA-10 rotor in the J2-HS Beckman centrifuge for 10 minutes at 4°C. The pellet was resuspended in 1 ml of ice-cold NET-NP buffer (150 mM NaCl, 5 mM EDTA [pH 8], 50 mM Tris-HCl

[pH 7.4] and 0.5% Nonidet-P40 [NP40] [Sigma]), transferred to an Eppendorf tube and centrifuged again at 5000 rpm in an Eppendorf microfuge for 5 minutes at 4°C. The pellet was resuspended in 200 µl of NET-NP buffer with 1 mM polymethylsulfonylfluoride (PMSF) and an equal volume of acid-washed glass beads (425 - 600 µm, Sigma) was added. This mixture was vortexed vigorously for 30 seconds and placed on ice for 5 minutes. This cycle was repeated 5 times and after the final cycle, the tube was centrifuged in an Eppendorf microfuge at 5000 rpm at 4°C for 30 seconds. The supernatant containing the yeast proteins was recovered and stored on ice or frozen at -86°C in 10 - 15% glycerol until further use.

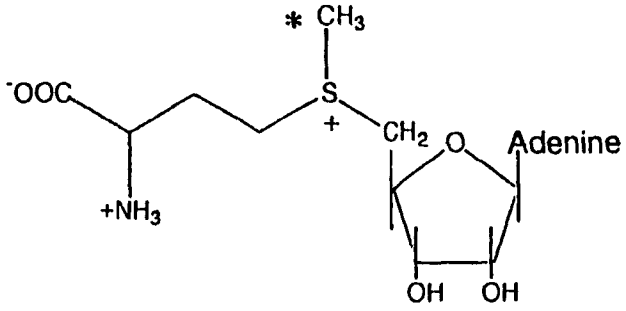
RESULTS

A. TRANSFER RNA-SPECIFIC METHYLTRANSFERASE ACTIVITY

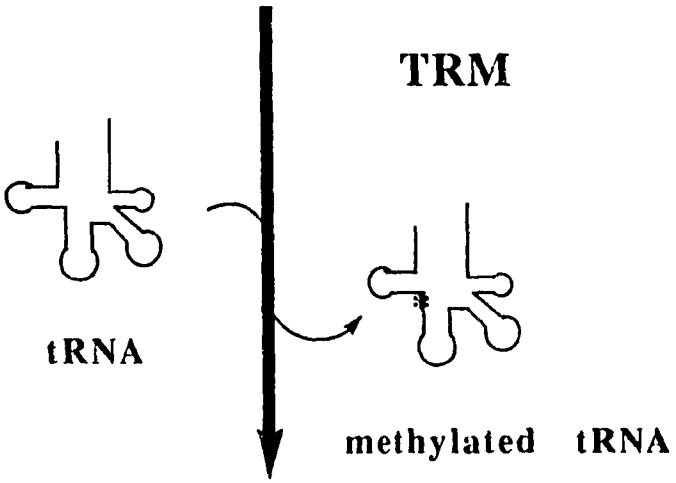
Specific tRNA methyltransferase activity was measured using a radioactive assay based on the transfer of tritiated methyl groups from S-adenosylmethionine (SAM) to tRNA (Figure 4). In order to account for the background of radioactive methyl groups transferred to bases in positions other than guanosine at position 26 (G_{26}), tRNAs containing N^2,N^2-dimethylguanosine at G_{26} were used. Using this system, we measured the difference in the levels of incorporated label in the two populations of tRNAs as an indication of specific incorporation of labelled methyl groups at G_{26} (Table 1). Since this assay can not distinguish between mono- and dimethylation at G_{26} we will refer to this activity as tRNA m^2G26-methyltransferase (TRM) activity indicating only that we are measuring methyl group incorporation at the undermethylated 2-amino position of the guanosine residue at position 26.

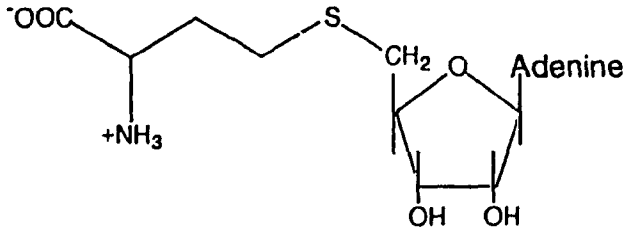
When the conditions used to assay the yeast enzyme (Ellis *et al.*, 1986) were used with DEAE-Sepharose purified lupin protein, little activity was seen (Table 2). Therefore, attempts were made to improve this assay for lupin extracts. When the amount of protein was increased from 100 μg per assay to 300 μg per assay, an approximately 3 - fold increase in incorporation of label with SN1015-2a and W303-1b tRNA was seen. Increasing the amount of tRNA

FIGURE 4: REACTION CATALYZED BY TRM



S-Adenosylmethionine (SAM)





S-Adenosylhomocysteine

TABLE 1: DEVELOPMENT OF TRM ACTIVITY ASSAY

Protein Source	Substrate supplied	Label incorporated (cpm)	TRM activity[3] (cpm)
W303-1b[1]	no tRNA	880	17 499
	W303-1b tRNA	924	
	SN1015-2a tRNA	18 423	
SN1015-2a[2]	no tRNA	437	116
	W303-1b tRNA	461	
	SN1015-2a tRNA	577	

1 The W303-1b protein extract contains an active N^2,N^2-dimethylguanosine-specific tRNA methyltransferase and its tRNAs have the N^2,N^2-dimethylguanosine modification at position 26.

2 The SN1015-2a protein extract contains a non-functional N^2,N^2-dimethylguanosine-specific tRNA methyltransferase and its tRNAs do not have the N^2,N^2-dimethylguanosine modification at position 26.

3 TRM (tRNA m^2G26-methyltransferase) activity is the difference between the incorporated label when SN1015-2a tRNAs are used as substrate and when W303-1b tRNAs are used as substrate.

TABLE 2: IMPROVEMENT OF TRM ACTIVITY ASSAY

protein (μg)	tRNA (μg)	volume (μl)	time (min)	[^{3}H]-SAM (μCi)	pH	W303-1b tRNA[1] (cpm)	SN1015-2a tRNA[2] (cpm)	TRM activity[3] (cpm)
100	10	130	30	0.30	7.5	140	201	61
300	10	130	30	0.30	7.5	456	608	152
300	100	130	30	0.30	7.5	445	607	162
300	100	150	30	0.30	7.5	454	586	132
300	100	150	60	0.30	7.5	820	1097	277
300	100	150	60	0.48	7.5	935	1396	461
300	100	150	60	0.48	8.0	681	1459	778

[1] Strain W303-1b contains an active N^2,N^2-dimethylguanosine-specific tRNA methyltransferase and its tRNA contain the modification N^2,N^2-dimethylguanosine at position 26.

[2] Strain SN1015-2a contains an inactive N^2 N^2-dimethylguanosine-specific tRNA methyltransferase and its tRNA do not contain the modification N^2,N^2-dimethylguanosine at position 26.

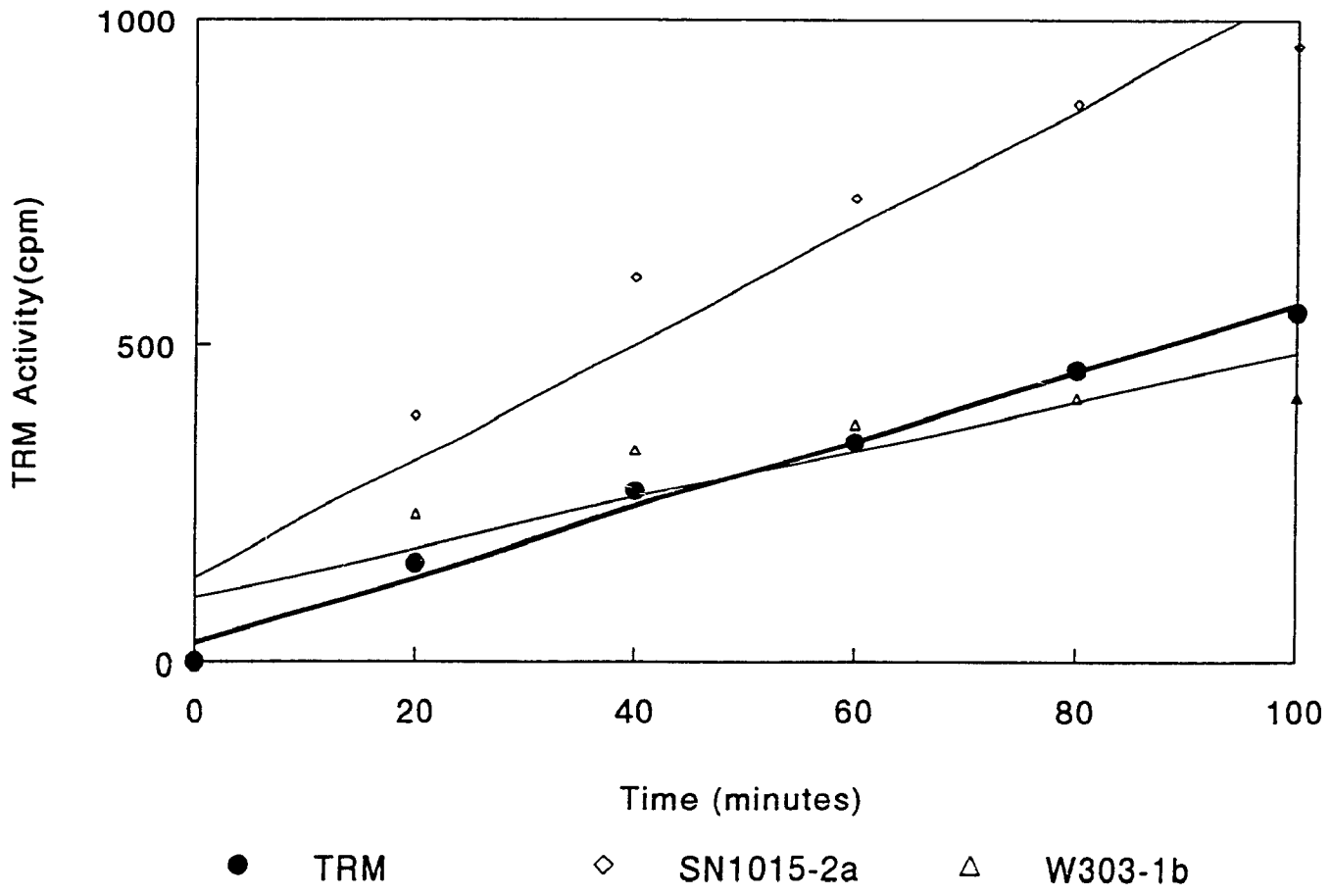
[3] TRM activity is the difference between the incorporated label when SN1015-2a tRNAs are used as substrate and when W303-1b tRNAs are used as substrate.

from 10 μg per assay to 100 μg per assay did not increase the label incorporated into either SN1015-2a or W303-1b tRNAs. Precipitating 150 μl of the reaction instead of 130 μl did not significantly alter the label incorporated into SN1015-2a or W303-1b tRNAs. Increasing the incubation time of the assay from 30 minutes to 60 minutes resulted in a 1.9 - fold improvement in the level of incorporated label into SN1015-2a and W303-1b tRNA. Increasing the amounts of radiolabelled [^{3}H]-SAM from 0.30 μCi to 0.48 μCi and non-radiolabelled SAM from 0.43 μg to 0.72 μg, showed only a 1.1 - fold increase in the label incorporated into W303-1b tRNA while it showed a 1.3 - fold increase in label incorporated into SN1015-2a tRNA. Perhaps the most interesting observation was that increasing the pH of the assay from 7.5 to 8 resulted in a 27 % decrease in incorporation into W303-1b tRNA but had little or no effect on incorporation of label into SN1015-2a tRNA.

Taken together, these changes resulted in increasing the incorporation of label into SN1015 tRNAs by greater than 7 - fold as compared to an increase of incorporation into W303-1b tRNAs of less than 5 - fold as compared with the initial yeast conditions. More significantly these alterations resulted in almost a 13-fold increase in the number of counts that could be attributed to tRNA m^2G26-specific methyl group transfers.

Using the assay conditions for optimal activity (row 7 of Table 2), time dependence kinetics for partially purified tRNA m^2G26-methyltransferase (TRM) showed that at 60 minutes the rate of production of radiolabelled tRNA was still linear (Figure 5), therefore, this incubation time and these assay conditions were

FIGURE 5: TIME DEPENDENCE KINETICS OF TRM ACTIVITY



used in all future assays.

B. *Lupinus albus* GRINDING

The total protein obtained from grinding one kilogram of dry *Lupinus albus* (lupin) seeds ranged from 10 - 15 g. When 400 g of lupin seeds were used approximately 4 g of protein was obtained indicating that the yield of protein from each grinding of lupin seeds was linear up to at least 1 kg.

C. AMMONIUM SULFATE FRACTIONS

1. FRACTIONS

Various ranges of ammonium sulfate fractionation steps including 0 - 40 and 40 - 60 %, 0 - 60%, 0 - 30 and 30 - 60%, 0 - 40 and 40 - 60%, 0 - 35 and 35 - 60% and finally 0 - 30, 30 - 50 and 50 - 70 % saturation (Table 3) were tried to optimize the recovery of TRM activity. Initially, greater than 90 % of the TRM activity was recovered in the 30 - 60 % saturation step. When this was repeated with a 30 - 50 % saturation step, not only was all of the activity recovered in this fraction but also it had the highest specific activity (Table 3), therefore, this step was used for all subsequent fractionations.

2. STABILITY

Before using the ammonium sulfate fractionated protein in further purification protocols, the effects of adding various protein stabilizing reagents to

TABLE 3: OPTIMIZATION OF AMMONIUM SULFATE FRACTIONATION OF CRUDE LUPIN PROTEIN

Ammonium Sulfate (%)	Protein (mg)	TRM Activity (cpm)	Recovered Activity (%)	Specific Activity (cpm/mg)
0-40[1]	1050	0.17×10^6	45	16
40-60	1784	0.2×10^6	55	114
0-60[1]	3500	1.3×10^6	100	362
0-30[1]	224	0.08×10^6	7	368
30-60	2100	1.1×10^6	93	535
0-35[1]	1294	1.4×10^6	37	1092
35-60	2817	2.4×10^6	63	856
0-30[2]	2827	0	0	0
30-50	4720	5.5×10^6	100	1177
50-70	7020	0	0	0

[1] protein from approximately 400 g of lupin seeds

[2] protein from approximately 1000 g of lupin seeds

the TME dialysis buffer in an attempt to avoid loss of TRM activity were tested. The following factors were tested on enzyme stability at 4°C: (1) low salt (20 mM NaCl), (2) EDTA (1 mM), (3) glycerol (10 %), (4) DTT (5 mM), (5) β-mercaptoethanol (5 mM) and (6) PMSF (1 mM) + EDTA (1 mM). TRM activity assays were performed 24, 48 and 96 hours after grinding and the results are shown in Table 4. The addition of 20 mM NaCl to TME buffer resulted in no significant loss of TRM activity but the label incorporated into both SN1015-2a and W303-1b tRNA decreased. The addition of 1 mM EDTA to TME buffer resulted in an apparent increase in TRM activity after 96 hours, however, this may be due to a major reduction in the level of label incorporated into W303-1b tRNA. The addition of glycerol (10%) to TME buffer resulted in an apparent increase in TRM activity although the levels of label incorporated into both SN1015-2a and W303-1b tRNA were decreased as compared to TME buffer alone. The addition of 1 mM PMSF to TME buffer stabilized the apparent levels of TRM activity obtained initially with TME buffer alone although incorporation of label into both SN1015-2a and W303-1b tRNAs dropped. The addition of 5 mM DTT had the same effect on TRM activity as TME buffer alone. The addition of 5 mM β-ME reduced the apparent TRM activity although the level of incorporation of methyl groups into both SN1015-2a and W303-1b tRNAs remained fairly constant over the 96 hours. The addition of 1 mM PMSF and 1 mM EDTA stabilized TRM activity and maintained high levels of label incorporated into both W303-1b and SN1015-2a tRNA. Although the level of TRM activity appeared to

TABLE 4: ABILITY OF CHEMICAL REAGENTS TO STABILIZE TRM ACTIVITY

reagent	tRNA	24 hours (cpm)	48 hours (cpm)	96 hours (cpm)
20 mM NaCl	SN1015-2a	4141 ± 207	3303 ± 10	2909 ± 68
	W303-1b	2578 ± 340	1824 ± 54	1669 ± 145
	TRM activity	1563 ± 274	1479 ± 32	1240 ± 107
1 mM EDTA	SN1015-2a	4244 ± 19	3812 ± 77	3540 ± 9
	W303-1b	3153 ± 89	2953 ± 119	1896 ± 201
	TRM activity	1091 ± 54	859 ± 98	1644 ± 105
10 % glycerol	SN1015-2a	3306 ± 68	3065 ± 83	3634 ± 75
	W303-1b	2577 ± 162	2316 ± 36	1818 ± 58
	TRM activity	729 ± 115	749 ± 60	1816 ± 67
1 mM PMSF	SN1015-2a	3652 ± 102	3043 ± 173	2733 ± 61
	W303-1b	2776 ± 130	1832 ± 58	1663 ± 144
	TRM activity	876 ± 116	1211 ± 116	1070 ± 103
5 mM DTT	SN1015-2a	4211 ± 346	3612 ± 163	3824 ± 175
	W303-1b	3151 ± 17	3061 ± 24	3071 ± 845
	TRM activity	1060 ± 182	551 ± 94	753 ± 510
5 mM β-ME	SN1015-2a	3377 ± 86	3083 ± 42	2908 ± 29
	W303-1b	2503 ± 143	2551 ± 194	2402 ± 36
	TRM activity	874 ± 115	532 ± 118	506 ± 33
1 mM PMSF + 1 mM EDTA	SN1015-2a	4376 ± 430	3562 ± 196	3761 ± 42
	W303-1b	2954 ± 108	2569 ± 187	2393 ± 43
	TRM activity	1422 ± 269	993 ± 192	1368 ± 42
TME	SN1015-2a	4132 ± 186	3335 ± 125	3873 ± 152
	W303-1b	2921 ± 176	2800 ± 44	3010 ± 563
	TRM activity	1211 ± 181	535 ± 85	863 ± 358

decrease at the 48 hour time point with TME buffer alone there is no significant difference between label incorporated into W303-1b or SN1015-2a tRNA, or apparent TRM activity between the 24 hour and 96 hour time points.

The effect of freezing and thawing on TRM activity was determined. Table 5 shows that numerous freezing and thawing cycles did not result in any significant loss of TRM activity.

D. COLUMN CHROMATOGRAPHY

1. DIETHYLAMINOETHYL-SEPHAROSE CHROMATOGRAPHY

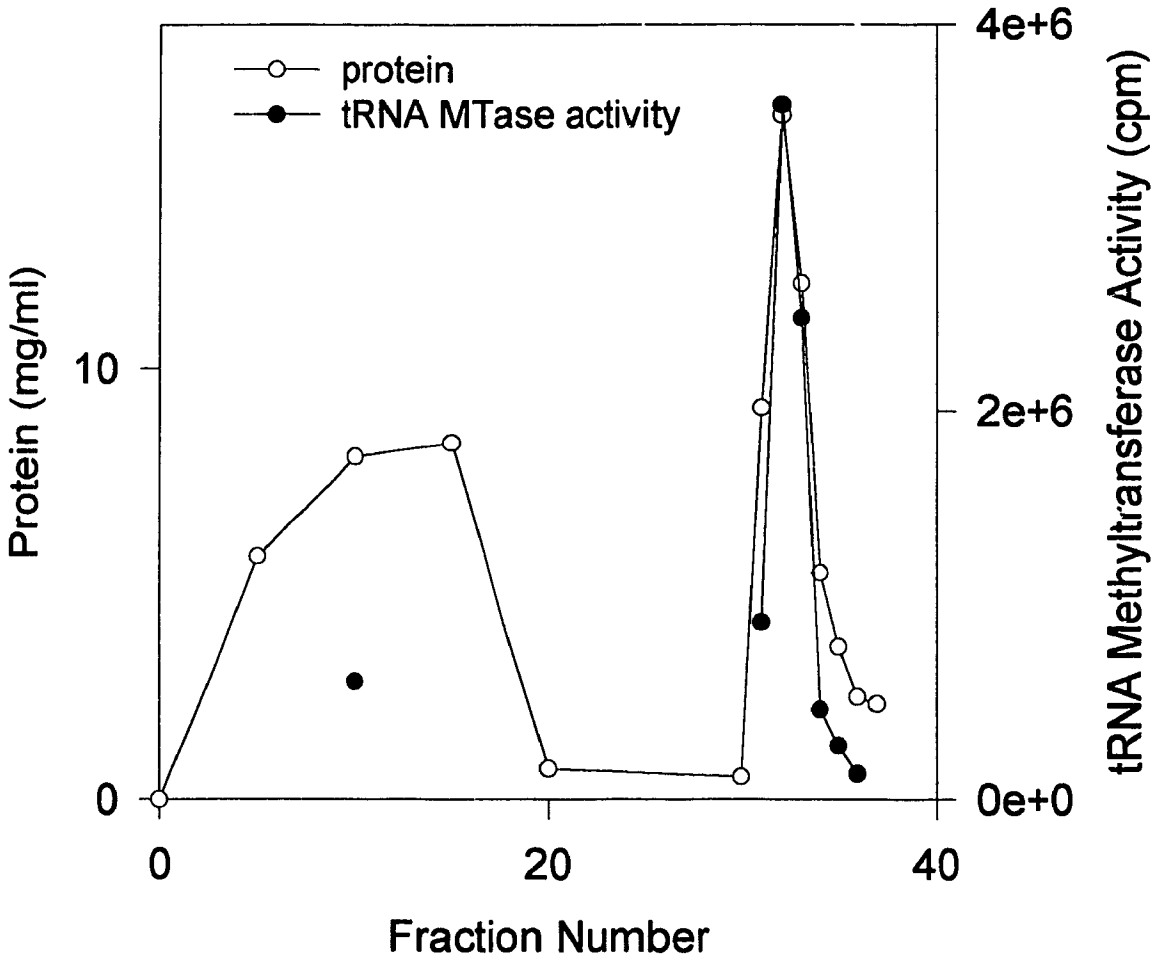
DEAE-Sepharose chromatography was chosen as the first chromatographic step in the purification of TRM. A previous report (Kwong, 1973) showed that wheat embryo tRNA methyltransferases containing TRM activity were retained on DEAE resin in the presence of 50mM Tris-HCl (pH 8) and could be eluted with 150 mM NaCl in 50 mM Tris-HCl (pH 8). Also, DEAE-Sepharose chromatography presented a fast and inexpensive way to accommodate the quantity of protein obtained after ammonium sulfate fractionation

Initially, binding of wheat embryo tRNA methyltransferases onto DEAE resin was repeated. To collect fractions as quickly as possible, DEAE-Sepharose Fast Flow was chosen instead of the DEAE-cellulose used by Kwong (1973). Wheat embryo proteins (40 - 60 % ammonium sulfate saturation step) were loaded onto the DEAE-Sepharose column in TME buffer and eluted with 150 mM NaCl in TME buffer (Figure 6). Initial activity assays were carried out

TABLE 5: EFFECT OF FREEZING AND THAWING OF TRM ACTIVITY

Times frozen and thawed	Activity with SN1015-2a tRNA (cpm)	Activity with W303-1b tRNA (cpm)	TRM Activity (cpm)
0	2378 ± 140	1356 ± 56	1022
1	2701 ± 67	1895 ± 244	806
2	2508 ± 146	1838 ± 20	670
3	2356 ± 42	1452 ± 117	904

FIGURE 6: WHEAT EMBRYO PROTEIN AND TRANSFER RNA METHYLTRANSFERASE ACTIVITY ELUTION FROM DEAE-SEPHAROSE CHROMATOGRAPHY



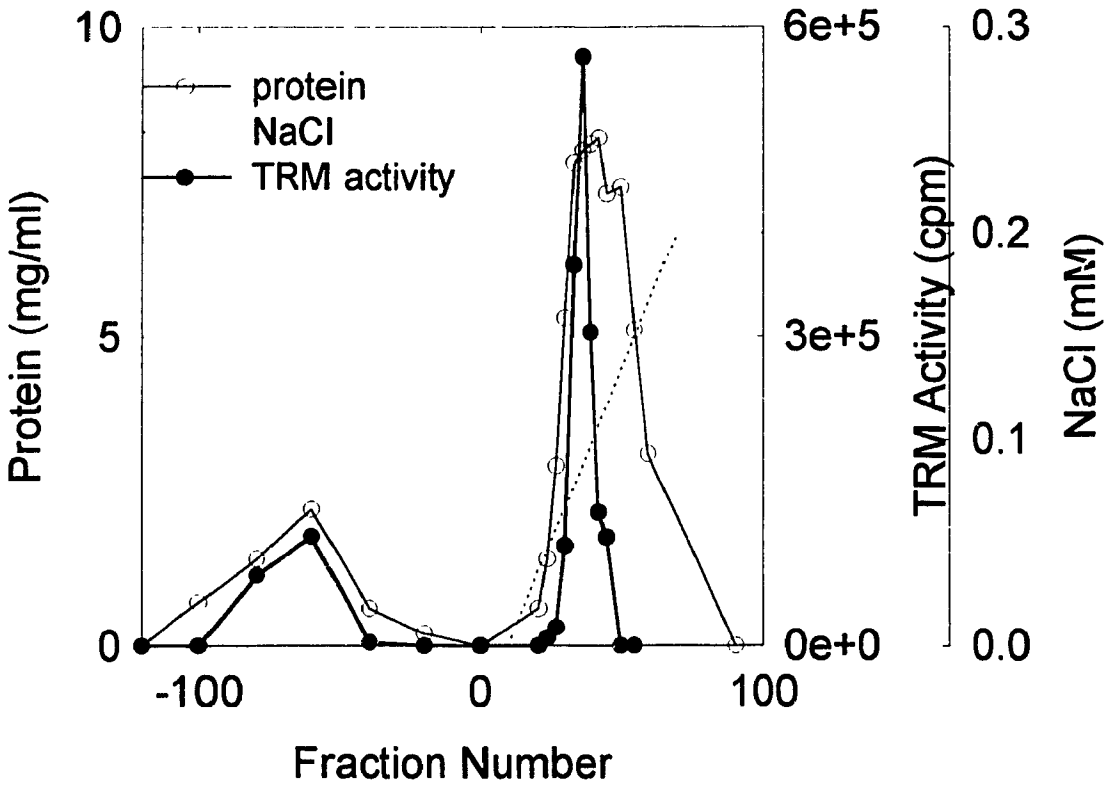
Approximately 3 g of wheat embryo protein (40 - 60 % ammonium sulfate saturation) was loaded onto the DEAE-Sepharose column at a flow rate of 7 ml/min in 50 mM Tris-HCl (pH 8) and 1mM DTT. The column was washed with this buffer until 150 mM NaCl was added at fraction 30. The fraction size was 12 mls. *E.coli* tRNA was used as substrate in the activity assays.

using *E.coli* tRNA as substrate. This measured total methyltransferase activity and was not specific for methylation at G_{26}. As expected from the data of Kwong (Kwong, 1973), a peak of tRNA methyltransferase activity eluted with the salt step. However, a point assayed for activity in the column flow-through also showed tRNA methyltransferase activity indicating that not all of the tRNA methyltransferases were retained on the DEAE-Sepharose column under these conditions.

In all further experiments these modifications were made: 1) protein was extracted from lupin seeds instead of wheat embryos because of the ease in obtaining protein from lupin because a procedure for obtaining crude protein had been developed in our laboratory (Shanmugam, 1994) and because initial experiments showed TRM activity in crude extract from lupin seeds 2) a 0 - 200 mM NaCl linear gradient was introduced to broaden the protein peak and 3) a TRM activity assay was developed for lupin protein so that it was no longer necessary to use *E.coli* tRNA as substrate. After these modifications the portion of the protein peak containing TRM activity could now be determined and it was apparent that some of the TRM activity was not retained on the DEAE-Sepharose column under these conditions (Figure 7).

Under our standard conditions (see Materials and Methods), 5 - 6 g of protein were loaded and the majority was retained on the DEAE-Sepharose column (Figure 7). Using the 0 - 200 mM NaCl linear gradient, TRM activity appeared to elute in a reasonably sharp peak between approximately 50 and

FIGURE 7: LUPIN PROTEIN AND TRM ACTIVITY ELUTION PROFILES FROM DEAE-SEPHAROSE CHROMATOGRAPHY



Approximately 6 g of lupin protein (30 - 50 % ammonium sulfate saturation) was loaded onto the DEAE-Sepharose column at a flow rate of 6 ml/min in TME buffer. The column was washed with 5 l of TME buffer before the 0 - 200 mM NaCl linear gradient was added at fraction 0. The fraction size was 12.5 mls.

100 mM NaCl while most of the protein appeared to elute between approximately 50 and 170 mM NaCl. Regardless of the amount of activity that was eluted from the gradient, fractions 30 - 40 (Figure 7) were pooled. Typically, approximately 50 % of recovered TRM activity was found in the flow through of the DEAE column (Figure 7).

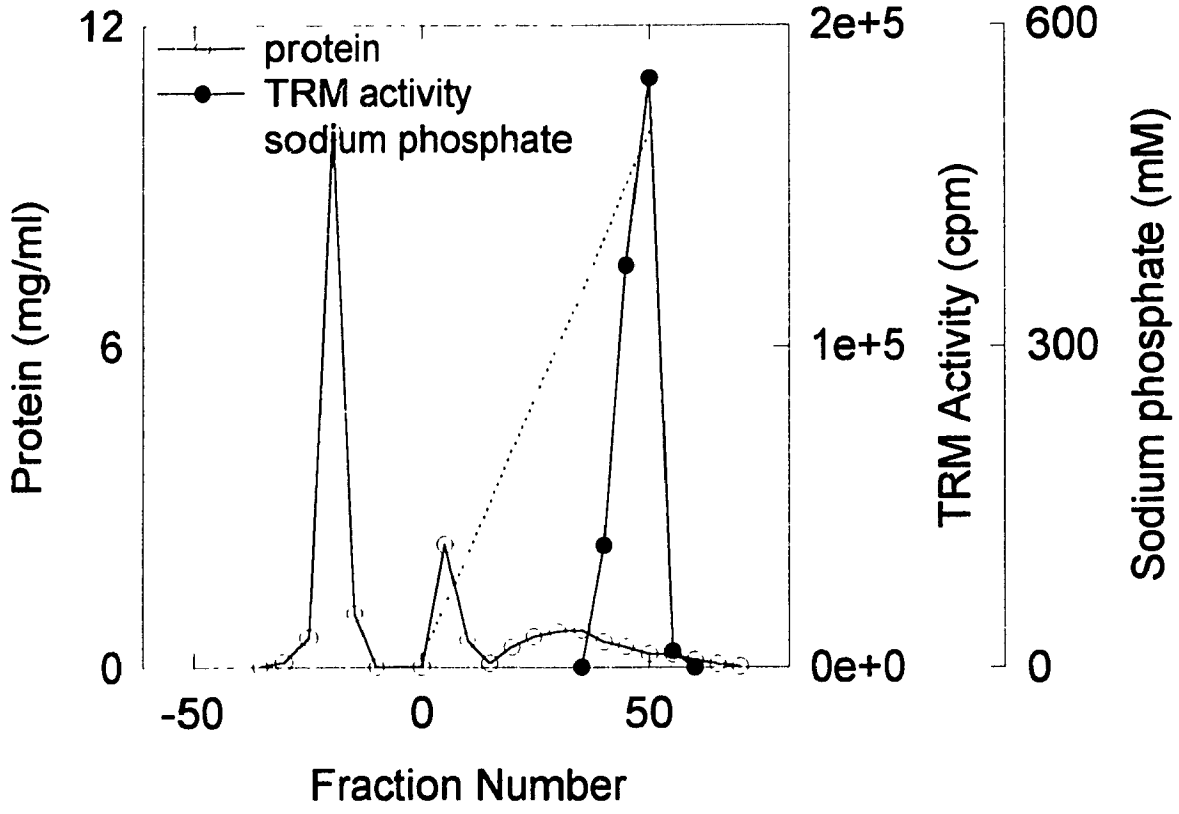
2. HYDROXYAPATITE CHROMATOGRAPHY

Because hydroxyapatite chromatography had proven useful in purifying tRNA nucleotidyltransferase from *Lupinus albus* in our lab (Shanmugam, 1994), it was chosen as the next chromatographic step. The pooled, active fractions eluted from the DEAE-Sepharose column were loaded directly onto the hydroxyapatite column in TME + NaCl buffer.

Because the TRM activity and protein elution profiles were consistent among different DEAE-Sepharose columns (Figure 7), the active fractions (#30 - 40) from the DEAE-Sepharose column were pooled and loaded onto the hydroxyapatite column prior to TRM activity assays on the individual fractions. This was done to avoid the loss of TRM activity seen when the protein was left at 4°C for 4 hours while protein and TRM activity assays were carried out (data not shown).

On average, approximately 1 - 1.3 g of pooled, active protein was loaded onto the hydroxyapatite column (Figure 8). Washing with TME buffer was carried out until the protein content in the collected fractions was below

FIGURE 8: LUPIN PROTEIN AND TRM ACTIVITY ELUTION PROFILES FROM HYDROXYAPATITE CHROMATOGRAPHY



Approximately 1.1 g of lupin protein eluted from the DEAE-Sepharose column was loaded onto the hydroxyapatite column in TME + NaCl buffer at a flow rate of 1 ml/min. The column was washed with TME buffer before the 10 - 500 mM sodium phosphate (pH 6.5) linear gradient was added at fraction 0. Additional 500 mM sodium phosphate (pH 6.5) was added at fraction 45. The fraction sizes were 12 mls during loading and 9.5 mls during the linear gradient.

detectable levels with the Bradford assay (OD_{595nm} less than 0 1) and adsorbed proteins then were eluted with a 10 - 500 mM sodium phosphate buffer (pH 6 5) linear gradient and an additional 60 mls (1 column volume) of 500 mM sodium phosphate buffer (pH 6.5).

To optimize elution of TRM activity from the hydroxyapatite column, different sodium phosphate (pH 6.5) linear gradients were tried: 10 - 400 mM, 100 - 400 mM, 250 - 500 mM, 200 - 500 mM and 10 - 500 mM (data not shown) and the optimum linear gradient was found to be 10 - 500 mM sodium phosphate This linear gradient was used in all subsequent hydroxyapatite columns. Step gradients of 0.7 M and 1 M sodium phosphate (pH 6 5) also were used after the linear gradients (data not shown) It was difficult to go higher than 500 mM sodium phosphate (pH 6.5) because of its insolubility at 4°C Crystals formed in the buffer and in the column damaging the resin and affecting the flow rate of the column.

No TRM activity was detected in protein that was not adsorbed by the hydroxyapatite column. Most of the absorbed protein eluted between 10 and 100 mM sodium phosphate buffer concentration (Figure 8) and TRM activity eluted between approximately 400 and 500 mM sodium phosphate buffer concentration, in a region of the gradient that contained less than 5 % of the protein that was loaded onto the column.

All of the sodium phosphate-eluted fractions (145 - 160) that showed TRM activity were pooled and dialyzed against 20 mM sodium phosphate buffer (pH

6) for 5 hours.

3. tRNA AFFINITY CHROMATOGRAPHY

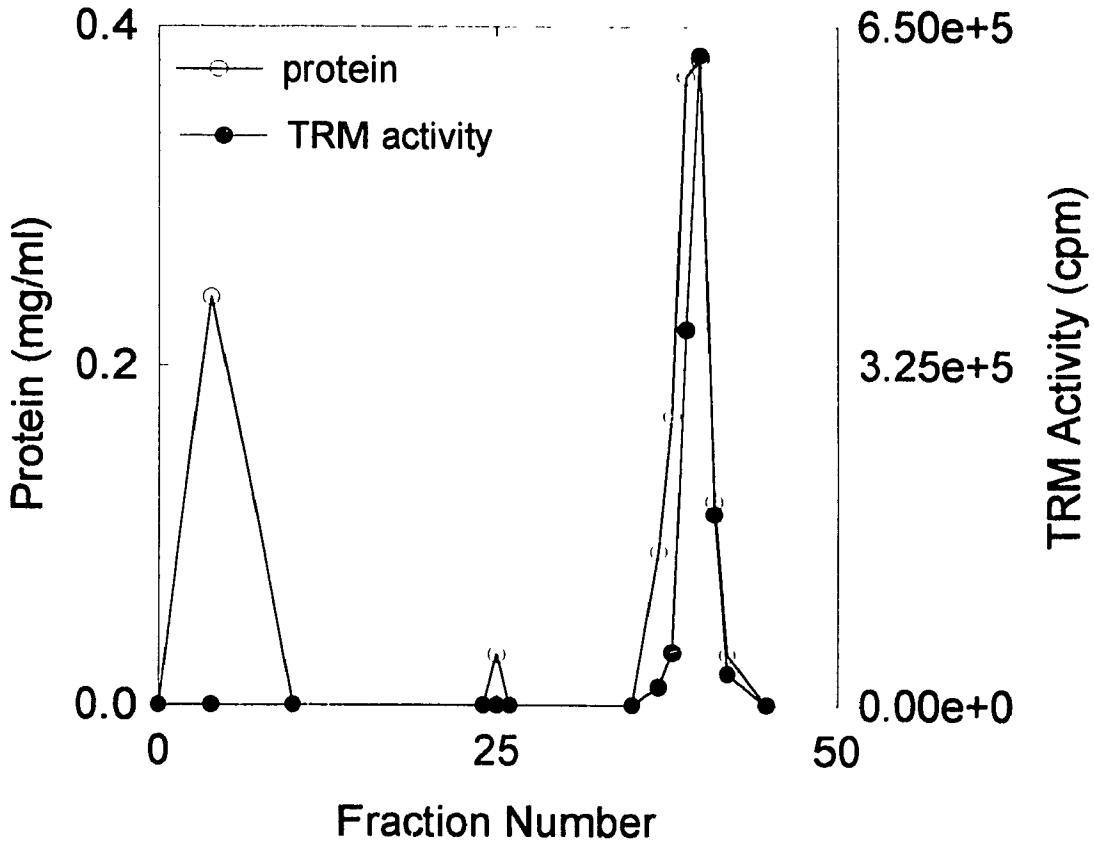
Transfer RNA affinity chromatography was chosen to follow hydroxyapatite chromatography because it was specific for tRNA-binding proteins and because the amount of total protein had been reduced in the DEAE and hydroxyapatite columns so that a small tRNA affinity column could be used.

The column was loaded with active, pooled fractions from the hydroxyapatite column which had been dialyzed against 20 mM sodium phosphate buffer (pH 6) for 5 hours. The column then was washed with 20 mM sodium phosphate until there was no detectable protein in the collected fractions with the Bradford assay (OD_{595nm} less than 0.1). Washing was continued with 50 mM Tris-HCl (pH 8.5) and subsequently with 250 mM NaCl in 50 mM Tris-HCl (pH 8.5) to elute TRM activity.

The majority of proteins were found in the wash and there was no TRM activity among these proteins (Figure 9). When the pH was changed from 6 to 8.5 by replacing the sodium phosphate buffer with Tris-HCl buffer little TRM activity was found although other proteins were eluted (Figure 9). The majority of recovered TRM activity was eluted with 250 mM NaCl in Tris-HCl buffer (Figure 9).

In order to optimize the elution of TRM activity from the tRNA affinity column, numerous salt gradients were tried: linear gradients of 0 - 300 mM, 0 -

FIGURE 9: LUPIN PROTEIN AND TRM ACTIVITY ELUTION PROFILES FROM tRNA-AFFINITY CHROMATOGRAPHY



Pooled, dialyzed active lupin protein (approximately 50 mg) eluted from the hydroxyapatite column was loaded onto the tRNA-affinity column in 20 mM sodium phosphate (pH 6) at a flow rate of 1 ml/min. Washing was continued with this buffer until 50 mM Tris-HCl (pH 8.5) buffer was added at fraction 20 and 250 mM NaCl was added at fraction 30. The sizes of the fractions were 12 mls during loading and washing and reduced to 2 mls during washing with 250 mM NaCl.

200 mM and 150 - 300 mM NaCl as well as step gradients of 20 mM, 100 mM, 250 mM and 150 mM and 250 mM NaCl (data not shown). Because none of these fractionation protocols resulted in increased recoveries of TRM activity or as great an increase in specific activity as simply using a 250 mM NaCl step gradient I continued to use only the 250 mM NaCl step.

All of the active fractions eluted with 250 mM NaCl from tRNA affinity chromatography were pooled and stored at -86°C in 10 - 15% glycerol until further use.

4. ADENOSINE-AFFINITY CHROMATOGRAPHY

The fact that there is an adenosine moiety in S-adenosylmethionine, one of the substrates in the reaction catalyzed by TRM, suggests that TRM may show affinity for adenosine-affinity resin. Because of this, an adenosine-affinity column was used as the next step.

Active protein totaling approximately 3 mg, eluted from two tRNA-affinity columns, was dialyzed against TME buffer for 4 hours and loaded onto the adenosine-agarose column. Most of the loaded protein was not bound to this column (Figure 10). Washing was continued with TME buffer containing 200 mM NaCl and some protein was eluted. Even less protein was eluted when 1 mM SAM was added to TME buffer containing 200 mM NaCl.

The majority of TRM activity did not bind to the column and was collected in the flow through (Figure 10). The largest increase in specific activity of TRM,

however, was observed with the first fraction eluted after addition of the 200 mM NaCl step. There was no TRM activity (measured after dialyzing out the SAM for 4 hours) in the fractions eluted with 1 mM SAM.

In order to determine whether the TRM activity that did not bind to the adenosine-affinity column was due to overloading this column, the protein that flowed through was loaded again onto the adenosine-affinity column (Figure 11). This time there was no TRM activity in the flow through and TRM activity was only eluted with the 200 mM NaCl washing step. There was no TRM activity (after 4 hours of dialysis in TME buffer) in the fractions eluted with 1 mM SAM.

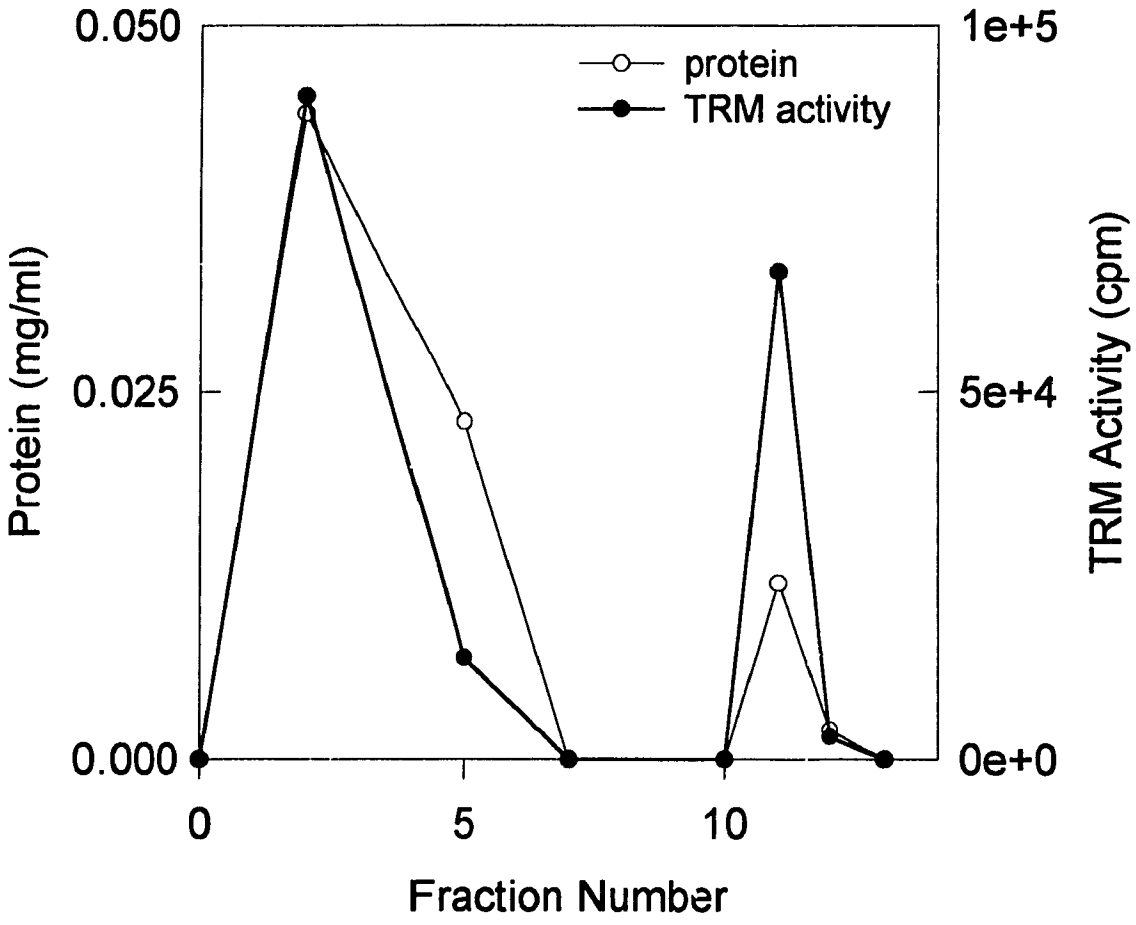
E. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

In order to verify the extent of purification of TRM, SDS-polyacrylamide gel electrophoresis was used. Figure 12 shows the decrease in the number of proteins during the purification steps such that the most purified lane contains the fewest proteins.

F. WESTERN ANALYSIS

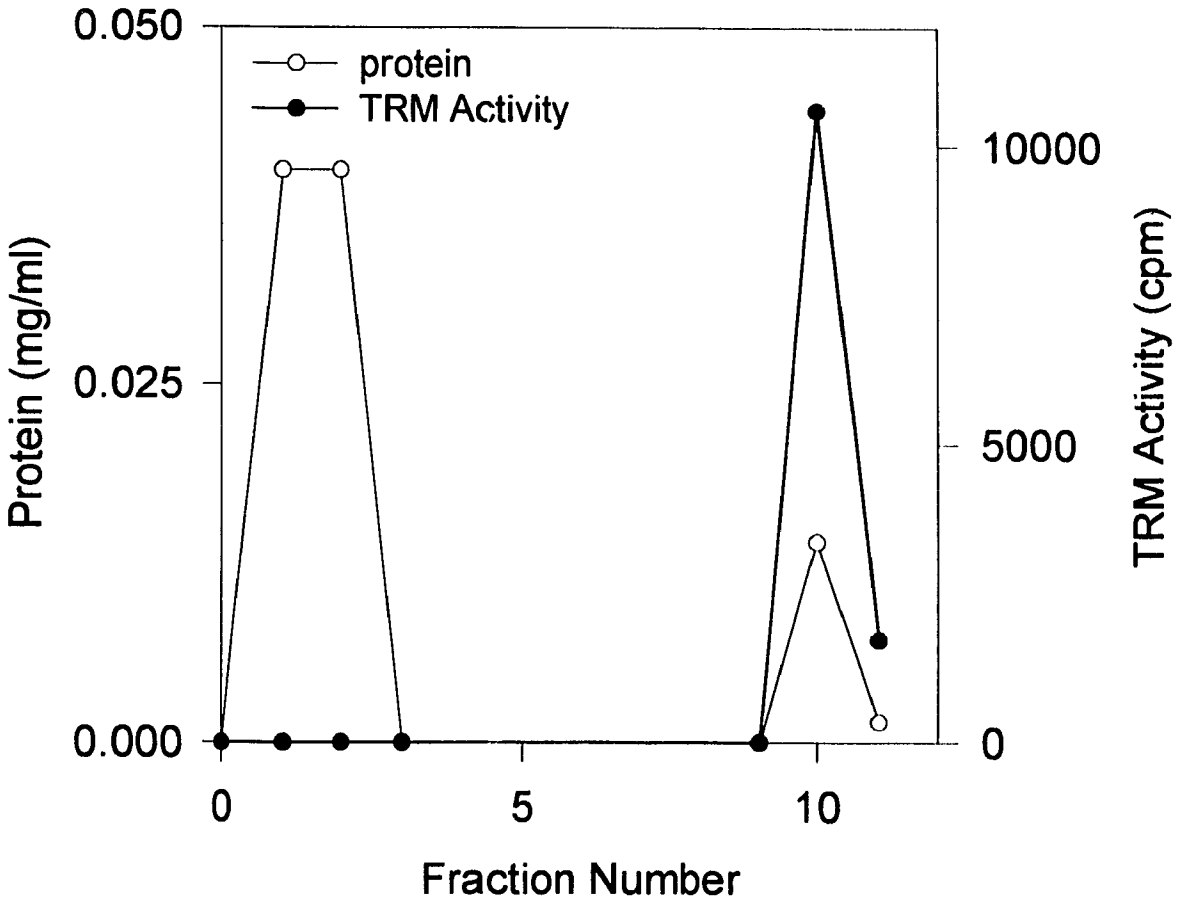
The results from the Western blot show that the yeast *TRM1* antibody cross-reacts with a protein at approximately 66 kDa in the most purified fractions eluted from the adenosine-affinity column (Figure 13). This antibody also cross-reacts with other proteins in the less pure fractions and with crude yeast proteins.

FIGURE 10: LUPIN PROTEIN AND TRM ACTIVITY ELUTION PROFILES FROM ADENOSINE-AFFINITY CHROMATOGRAPHY



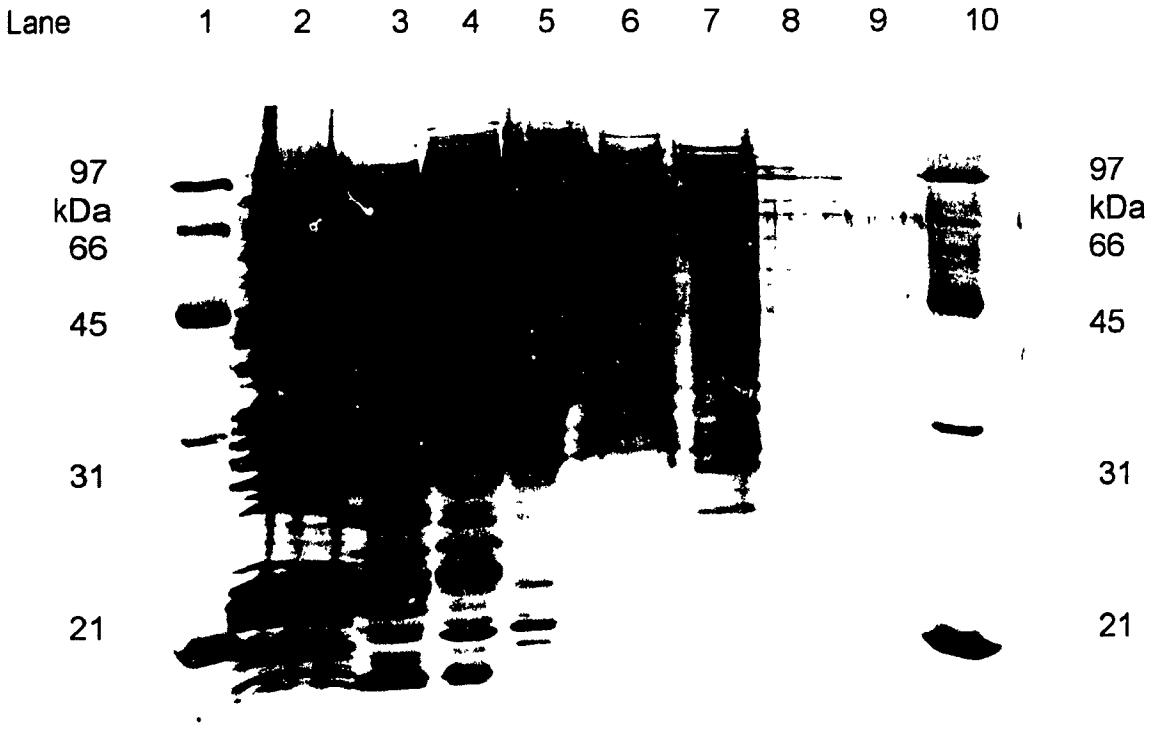
Approximately 3 mg of pooled, dialyzed active protein from two tRNA-affinity columns were loaded onto the adenosine-affinity column in TME buffer at a flow rate of 1 ml/min. Washing was continued with TME buffer until 200 mM NaCl in TME buffer was added at fraction 10. When 1 mM SAM was added to the 200 mM NaCl in TME buffer at fraction 20, the amount of protein eluted was below the limit of detection. The fraction sizes were 12 mls during loading and washing and 1 ml during washing with 200 mM NaCl.

FIGURE 11: LUPIN PROTEIN AND TRM ACTIVITY ELUTION PROFILES AFTER LOADING FLOW THROUGH FROM PREVIOUS COLUMN ONTO A SECOND ADENOSINE-AFFINITY COLUMN



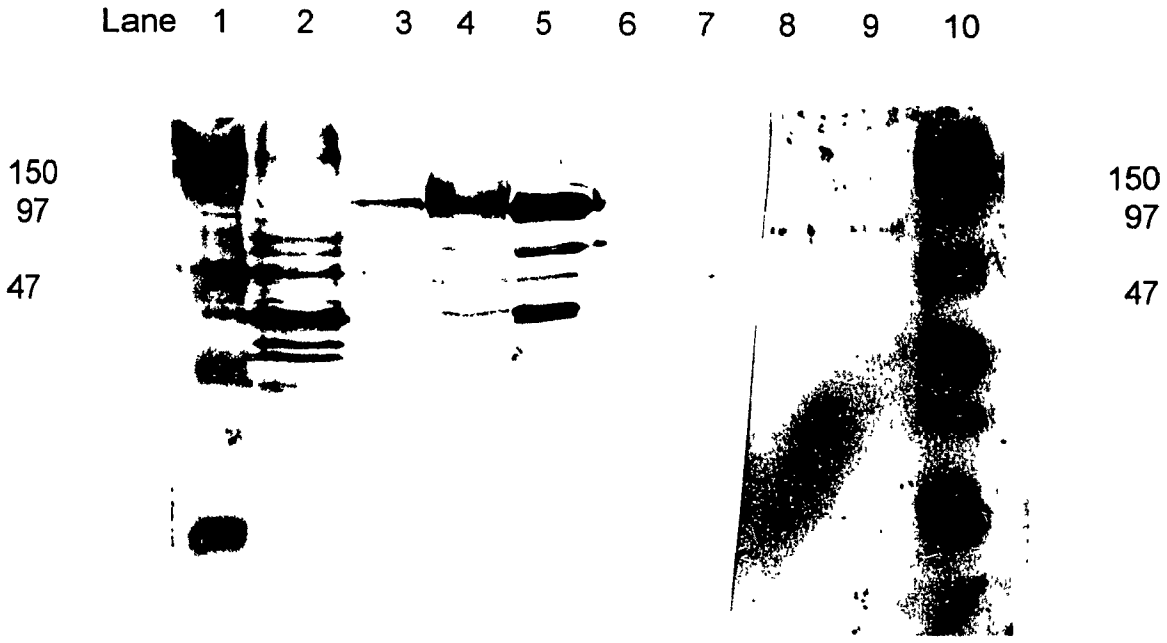
The protein that flowed through the previous adenosine-affinity column that had TRM activity was reloaded onto the adenosine-affinity column in TME buffer at a flow rate of 1 ml/min. Washing was continued with TME buffer until 200 mM NaCl in TME buffer was added at fraction 10. The fraction sizes were 12 mls during loading and washing and 1 ml during washing with 250 mM NaCl.

FIGURE 12: SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS SHOWING THE PURIFICATION OF LUPIN TRM



Silver stained 10% SDS-polyacrylamide gel. Lane 1 and Lane 10 - low range molecular weight markers. Lane 2 - crude yeast protein. Lane 3 - crude lupin protein (20 μg). Lane 4 - 30 - 50 % ammonium sulfate step (20 μg). Lane 5 - pooled, active fractions eluted from DEAE-Sepharose column (20 μg). Lane 6 - pooled, active fractions eluted from hydroxyapatite column (2.7 μg). Lane 7 - pooled, active fractions eluted from tRNA-affinity column (1.8 μg). Lane 8 - active fraction eluted from adenosine-affinity column with 200 mM NaCl. Lane 9 - inactive fraction eluted from the adenosine-affinity column with 1 mM SAM.

FIGURE 13: WESTERN BLOT ANALYSIS OF LUPIN TRM WHEN RABBIT SERUM CONTAINING YEAST *TRM1* ANTIBODY IS USED AS PROBE



Western blot analysis of lupin protein with yeast *TRM1* antibody. Lane 1 and Lane 10: prestained molecular weight markers. Lane 2 - inactive fraction eluted from the adenosine-affinity column with 1 mM SAM. Lane 3 - active fraction eluted from adenosine-affinity column with 200 mM NaCl. Lane 4 - pooled, active fractions eluted from tRNA-affinity column. Lane 5 - pooled, active fractions eluted from hydroxyapatite column. Lane 6 - pooled, active fractions eluted from DEAE-Sepharose column. Lane 7 - 30 - 50 % ammonium sulfate step. Lane 8 - crude lupin protein. Lane 9 - crude yeast protein Lanes 1-3 were stained twice as long as lanes 4-10.

G. PURIFICATION OF tRNA m^2G26-METHYLTRANSFERASE

An attempt to tabulate the data obtained from the purification of TRM is shown in Table 6. From this table, it can be seen that an overall 6153 - fold enrichment of TRM activity after ammonium sulfate fractionation and DEAE-Sepharose, hydroxyaptite, tRNA-Sepharose and adenosine-affinity chromatography was achieved although very little active protein was recovered.

TABLE 6: PURIFICATION OF TRM FROM LUPIN SEEDS

Purification Step	Protein (mg)	Specific Activity (cpm/mg)	Enrichment	TRM Activity (cpm)	Yield (%)
crude	10 536	0.9×10^3	1	948×10^4	100
30 - 50% ammonium sulfate	6015	1.4×10^3	1.6	842×10^4	89
DEAE	1140	2.5×10^3	2.8	285×10^4	30
HA	49	35.5×10^3	39	174×10^4	18
tRNA	2.5	355×10^3	394	89×10^4	9
AA[1]	0.005	5538×10^3	6153	2.8×10^4	0.3

[1] Results from 200 mM NaCl wash step from Figure 10.

DISCUSSION

An enzyme, tRNA m^2G26-methyltransferase (TRM), that catalyzes the methylation of guanosine residues at position 26 in tRNA has been purified from *Lupinus albus* through a combination of chromatographic techniques and an assay system to specifically monitor this reaction has been developed for *Lupinus albus*.

A. MEASUREMENT OF tRNA m^2G26-METHYLTRANSFERASE ACTIVITY

Initially, *E.coli* tRNAs were used as substrate in the TRM activity assays because *E.coli* lacks the enzyme required to synthesize N^2,N^2-dimethylguanosine at G_{26} (Phillips and Kjellin-Stråby, 1967). To date a minimum of seven methylated positions have been identified in eubacteria but a minimum of twenty-three methylated positions have been characterized in eukaryotes (Figure 3 and Björk and Kohli, 1990). Therefore, it became evident that there was a problem in using *E.coli* tRNA as substrate for our assays since *E.coli* tRNAs contain many unmethylated nucleotides which may be methylated using our lupin extracts. To use *E.coli* tRNA as a specific substrate for tRNA m^2G26-methyltransferase, one would have to hydrolyze the tRNAs to mononucleotides and do thin layer chromatography (TLC) to show specific methylation at guanosine residues. However, this would be quite a lengthy process involving

an activity assay, isolation of tRNA, hydrolysis of tRNA, TLC and quantitating the spots on the TLC plate. Therefore, to avoid this, an activity assay was developed that could specifically measure methylation at G_{26} by lupin extracts.

Ideally, fully modified lupin tRNA which lacked only m^2G26 would be used as substrate. No such substrate was available, however, an assay system that specifically measures the methylation of the guanosine residue at position 26 of yeast tRNA is available (Ellis *et al.*, 1986). In this assay, tRNA isolated from the yeast strain SN1015-2a are used. This strain lacks a functional N^2,N^2-dimethylguanosine-specific tRNA methyltransferase enzyme and its tRNAs are not modified at position 26. In order to account for methylation at positions other than G_{26} in tRNAs isolated from SN1015-2a, tRNAs that contain N^2,N^2-dimethylguanosine are used. The yeast strain, W303-1b contains an active N^2,N^2-dimethylguanosine-specific tRNA methyltransferase enzyme which modifies tRNA at G_{26} to yield N^2,N^2-dimethylguanosine. Therefore, in theory, all of the positions that get methylated in SN1015-2a tRNAs using lupin extracts should also get methylated in W303-1b tRNA except for the guanosine at position 26 which is methylated in this yeast strain. The difference in the level of incorporation of radiolabelled methyl groups into SN1015-2a tRNAs and into W303-1b tRNAs after incubation with lupin extract should define methylation at G_{26}.

Table 1 shows the results obtained when tRNAs isolated from either strain SN1015-2a or strain W303-1b were used as substrate in TRM activity

assays using protein extracts prepared from W303-1b or from SN1015-2a. There was a 2000 % increase in the label incorporated into SN1015-2a tRNA as compared to the label incorporated into W303-1b tRNAs when W303-1b protein extract was used as the source of TRM activity. However, there was only a 25 % increase in the label incorporated into SN1015-2a tRNAs as compared to W303-1b tRNAs when SN1015-2a protein extract was used as the source of TRM activity. The level of label incorporated in the assays without tRNA and with the fully modified W303-1b tRNA could be due to the fact that the crude protein extract itself contained tRNAs that had not yet been fully modified so that the tRNA methyltransferases present in this crude extract could also methylate these tRNAs during the activity assay. By subtracting the label incorporated into W303-1b tRNA from that incorporated into SN1015-2a tRNA, one can control for non-specific methylation. This assay system shows a much greater incorporation of radiolabelled methyl groups into SN1015-2a tRNA than into W303-1b tRNA when yeast protein is used as the source of enzyme (Table 1). When this procedure was used with lupin extracts, the level of label incorporated into both W303-1b and SN1015-2a tRNAs was significantly less (Table 2, row 1). This may be due to the assay conditions not being optimized for the lupin enzyme, other enzymes using up the radiolabelled methyl groups (generating products that are not acid precipitable), or to degradation of the tRNA substrates by nucleases present in the lupin extracts Modifications were made to this procedure to increase the label incorporated with the lupin enzyme. The results

from these modifications are shown in Table 2.

When the amount of protein from the yeast standard conditions (100 μg) was increased to 300 μg per assay (Table 2, row 2), the level of label incorporated into SN1015-2a tRNA and into W303-1b tRNA increased 3 fold. The calculated TRM activity was also increased about 3 - fold. This increase in the incorporated label was expected because increasing the protein in the activity assay could simply result in more enzyme being present such that more product can be formed in the same time period Since an increase in TRM activity was obtained, the protein level was maintained at 300 μg for the remaining assays.

When the amount of tRNA was increased from 10 μg per assay to 100 μg per assay (Table 2, row 3), no significant increase in the amount of label incorporated into SN1015-2a tRNA or into W303-1b tRNA was observed. This result indicated that the level of tRNA was already saturating at 10 μg and again argues against degradation of tRNAs during the assay as a reason why low label incorporation was observed. However, the amount was kept at 100 μg per assay to ensure that there was sufficient tRNA available for competing reactions such as degradation.

Doubling the incubation time of the assay from 30 minutes to 60 minutes resulted in a 2-fold increase in TRM activity (Table 2, row 5). Allowing the enzyme to react twice as long meant that twice as much product could be formed if neither of the substrates were limiting and the level of label incorporated into

SN1015-2a tRNAs and into W303-1b tRNAs would increase. Time-dependence kinetics were done to determine if the reaction had gone to completion within the 60 minute time period of the assay. These results (Figure 5) showed that over a period of 100 minutes, the rate of product formation was still linear with time. This indicated that during the 60 minute incubation period, the reaction had not gone to completion. Therefore, it was acceptable to increase the incubation time to 60 minutes.

Increasing the amount of [^{3}H]-SAM to 0.48 μCi per assay from 0.30 μCi per assay and the amount of SAM from 0.43 μg per assay to 0.72 μg per assay resulted in an approximately 14 % increase in the amount of label incorporated into W303-1b tRNAs but a greater than 27 % increase in label incorporated into SN1015-2a tRNA (Table 2, row 6). From these data, the calculated TRM activity was increased by approximately 66 %. The levels of [^{3}H]-SAM and SAM were not saturating during the assay, therefore, increasing their level led to increases in the label incorporated into SN1015-2a tRNA and into W303-1b tRNA. Different increases in the level of label incorporated were seen with SN1015-2a and W303-1b tRNA because the different tRNA methyltransferase enzymes that may be present in this extract may have different Km's for SAM. The 27 % increase in incorporation seen with SN1015-2a tRNA suggests a major increase in TRM activity in addition to these other tRNA methyltransferases. The 14 % increase in incorporation seen with W303-1b tRNA was due to an increase in the activity of all tRNA methyltransferases.

Radioactive decay of [^{3}H]-SAM does not significantly effect the apparent level of label incorporated between experiments because its half-life is 12.5 years (Boyer, 1993). However, SAM and [^{3}H]-SAM are not very stable in aqueous solutions and have short chemical half-lives of approximately 24 hours at 37°C. This short chemical half-life, however, should not significantly affect the levels of label incorporated between experiments because the length of the activity assay is only 60 minutes. This may have affected the level of SAM or [^{3}H]-SAM that was added to each assay after storage at -20°C.

Changing the pH of the assay from 7.5 to 8 yielded a 4 % increase in the level of label incorporated into SN1015-2a tRNA and a 27 % decrease in the level of label incorporated into W303-1b tRNA. Based on these results the apparent TRM activity was increased by 69 % (Table 2, row 7). The decrease in the level of label incorporated into W303-1b tRNA may be due to the fact that some of the other tRNA methyltransferases in the lupin extract function less efficiently at this pH. The increase in the level of incorporated label using SN1015-2a tRNA may be due to the fact that in this assay the pH was closer to the pH optimum of this enzyme. Similar results from pH studies were obtained by Kwong (1975) who showed that an increase in tRNA m^2G26-methyltransferase activity was selectively measured over other tRNA methyltransferases at pH 8.5 when DEAE-purified protein from wheat embryos was used.

Overall, there was a 12.8 - fold increase in the label incorporated using

the improved conditions. With this improved method, a significant difference between the levels of label incorporated with SN1015-2a tRNA and with W303-1b tRNA was observed which made it easier to identify active fractions during purification.

B. *Lupinus albus* GRINDING

Initially, wheat embryos were used as a source of TRM because this enzyme had been partially characterized in wheat embryos (Kwong, 1973). However, because this procedure was time-consuming and gave low yields of protein (approximately 6 g of protein from 30 kg of wheat seeds) an alternative procedure was developed. Between 10 and 15 g of protein could be obtained from 1 kg of lupin seeds in approximately 2 hours. Therefore, lupin was used as a source of TRM in all subsequent procedures.

C. AMMONIUM SULFATE FRACTIONS

1. FRACTIONS

Ammonium sulfate fractionation was used for two reasons, firstly, it concentrated the protein so that it reduced the amount of time required to load the DEAE-Sepharose column and secondly, it served as an initial purification step because it eliminated some contaminating proteins.

To determine which was the most effective fractionation, the percentage of TRM activity and the specific activity of each fractionation step was compared.

The results in Table 3 indicate that the optimum conditions were found to be 30 - 50 % ammonium sulfate saturation because most (if not all) of the TRM activity was precipitated in this step and this step resulted in the highest specific activity.

Although the relative amounts of TRM activity and protein were not comparable between grindings (Table 3) this was because the TRM assay conditions varied between experiments. The highest level of activity correlates with the improved assay procedure described previously. While it is difficult to compare results between different experiments this should not effect the interpretation of the results within any one experiment.

2. STABILITY OF tRNA m^2G26-METHYLTRANSFERASE ACTIVITY

Initially, the TRM enzyme lost significant activity when stored at 4°C (data not shown). In order to complete each chromatographic step during the purification procedure without losing most of the TRM activity, the ability of different chemical reagents to enhance the stability of TRM in TME buffer was tested. Some of the factors that may result in loss of enzyme activity include (1) protein denaturation (2) modification of the active site and (3) proteolysis (Scopes, 1987). Protein denaturation can be avoided if the pH of the buffer is kept within the physiological pH of the cell. The pH of TME buffer was kept at 8 because it is close to the pH of the lupin cellular environment. Also, TRM was found to be more active at this pH and binds to DEAE-Sepharose at this pH. The active site of the enzyme can be destroyed if it is chemically modified. The

addition of reducing agents such as DTT and β-ME help to keep exposed sulfhydryl groups on the enzyme or in the active site in their reduced state. DTT maintained the same levels of TRM activity as TME buffer alone and β-ME (5 mM) resulted in decreased TRM activity. It is known that β-ME should be replenished every 24 hours because it is readily oxidized in the presence of oxygen and can subsequently inhibit enzyme activity (Scopes, 1987). PMSF and EDTA were added to minimize the activity of proteolytic enzymes. PMSF is known to permanently inactivate the action of serine proteases and some carboxypeptidases and EDTA inhibits metalloproteases by chelating cations that are required for their activity. Either PMSF or EDTA alone did not stabilize TRM activity but together they were effective in maintaining TRM activity. The presence of glycerol resulted in an apparent increase in TRM activity over 96 hours. Glycerol was added to increase protein stability because it decreases the effective water concentration around the protein so that freezing and thawing does not affect the conformation of the active site (Scopes, 1987). The addition of low concentrations of NaCl to increase the ionic strength of the buffer in order to mimic the cellular ionic strength (Scopes, 1987) seemed to stabilize TRM activity while the activity of other tRNA methyltransferases decreased with time in its presence. None of these components were included in any subsequent purification procedures because none showed any significant improvements in the stability of TRM activity.

The effects of freezing and thawing on TRM activity were studied because

at times during the purification procedure it was necessary to freeze the active protein fractions before proceeding and other tRNA methyltransferases were not active after storage at -70°C (Chan and Fraser, 1972, Agris *et al.*, 1974, Wierzbicka *et al.*, 1975, Glick and Leboy, 1977,). These results are shown in Table 5 It was seen that as many as three short-term freezing and thawing cycles did not significantly alter TRM activity. Therefore, it was decided that TRM could be frozen and thawed during the purification procedure.

D. COLUMN CHROMATOGRAPHY

1. DEAE-SEPHAROSE CHROMATOGRAPHY

DEAE-Sepharose resin is composed of positively charged diethylaminoethyl groups covalently linked to Sepharose and binds negatively charged macromolecules (Scopes, 1987). The binding ability of these macromolecules depends on their net charge.

DEAE-Sepharose resin was used in the purification of TRM because other tRNA methyltransferases were previously fractionated with DEAE resin (Kwong, 1975, Pope and Reeves, 1978, Greenberg and Dudock, 1979, Keith *et al.*, 1980, Reinhart *et al*, 1986, Rjalmarsson *et al.*, 1983). The results of DEAE-Sepharose chromatography of wheat embryo proteins are shown in Figure 6 These results indicated that although some tRNA methyltransferases were bound to the DEAE-Sepharose resin and could be eluted with 150 mM NaCl, others did not bind to the column. However, at this time in the purification

procedure, total tRNA methyltransferase activity using *E.coli* tRNAs was being measured, therefore, the amount of TRM bound to the column was not accurately determined These results are comparable to others that show tRNA methyltransferases bound to DEAE resin and eluted with differing salt concentrations N^2,N^2-dimethylguanosine-specific tRNA methyltransferases from *Tetrahymena pyriformis* (Reinhart *et al.*, 1986) and HeLa cells (Agris *et al.*, 1974) were both fractionated using DEAE chromatography.

Once a method for specifically measuring TRM activity was developed, the region of the tRNA methyltransferase peak that contained TRM activity was identified These results (Figure 7) showed that TRM activity eluted at the beginning of the tRNA methyltransferase activity peak.

There was variation in the amount of TRM that was adsorbed onto the DEAE-Sepharose column The adsorbed TRM ranged from a low of 20 % of the recovered TRM activity to a high of 80 % of the recovered TRM activity. At least two factors could explain this variation: (1) the pH of the Tris-HCl buffer used or (2) the presence of two or more TRM enzymes in lupin.

The pH of Tris-HCl buffer is very temperature dependent (Stevens, 1992). A temperature change of 10°C results in a 0.3 unit change in the pH. During the course of this work, the preparation of the Tris-HCl buffer was changed. Initially, the pH was adjusted at room temperature, thus the 20°C change between pH adjustment at 22 - 25°C and use at 4°C could result in a pH increase of approximately 0.6 pH units. Therefore, the actual pH at which the protein was

loaded onto the column was approximately 8.6 instead of 8 When this Tris-HCl buffer was used, 80 % of recovered TRM activity was bound to the DEAE-Sepharose column (data not shown). When the pH of the Tris-HCl buffer was prepared at 4°C, approximately 50% of the recovered TRM activity was retained by the DEAE-Sepharose column (Figure 7). These results indicate that an increase in the pH of the Tris-HCl buffer from 8 to 8 6 could result in an increase in the net negative charge on the protein causing it to bind more efficiently to the DEAE-Sepharose resin When a Tris-HCl buffer compatible pH electrode was used and the pH adjusted to 8 at 4°C, approximately 20 % of the recovered TRM activity was bound to the DEAE-Sepharose column (data not shown) This indicates that at pH 8 the net charge on the protein is less negative causing it to bind less efficiently than at higher pH.

Another possible explanation of the variation observed with binding of TRM to the DEAE-Sepharose column could be the presence of more than one TRM enzyme in lupin extracts, i.e., different protein preparations may contain various amounts of these different enzymes. As mentioned earlier, this TRM activity assay does not distinguish between monomethylation and dimethylation of G_{26}, therefore, it is possible that lupin contains two TRM enzymes, one which catalyzes the monomethylation and another which catalyzes the dimethylation This has been proposed to occur with higher eukaryotes (Edqvist *et al.*, 1992) but separate enzymes have not yet been identified. These different enzymes, may have different primary structures thus resulting in different DEAE-

Sepharose-binding capabilities. In addition, preliminary data suggests that TRM activity is present in both crude cell and wheat embryo mitochondrial extracts (data not shown). Therefore, it is possible that these two activities represent cytosolic and mitochondrial enzymes. Another enzyme may also exist in the chloroplast because N^2,N^2-dimethylguanosine at position 26 has been seen in tRNAs isolated from the cytosol, the mitochondria and the chloroplast of plants (Sprinzl *et. al.*, 1991). In all, there is a possibility that there are at least three TRM enzymes in lupin, one for each of the tRNA-containing compartments, and possible more if different enzymes are responsible for mono and dimethylation of guanosines in each of these compartments Grinding of lupin seeds may break open the organelles and contaminate the cytosolic protein extract. However, in yeast, the only other well studied system, a single *TRM1* gene codes for the enzyme that is responsible for the mono and dimethylation activities measured in both the cytosol and the mitochondrion (Ellis *et al.*, 1986).

When TRM activity eluted from a DEAE-Sepharose column with a 0 - 200 mM NaCl linear gradient was loaded onto a second DEAE-Sepharose column, all of this activity was retained by the column and eluted only with the NaCl linear gradient. Since other experiments have shown that the first DEAE-Sepharose column was not overloaded, these results indicate that TRM not retained on the first DEAE-Sepharose column may be a different TRM isozyme than that which was retained on the DEAE-Sepharose column.

The active fractions from DEAE-Sepharose chromatography were pooled

and loaded immediately on the hydroxyapatite column in the same buffer (TME and NaCl) that was used to elute TRM from the DEAE-Sepharose column This was done because TRM was unstable at this point in the purification procedure and had to be processed quickly (data not shown). When the pooled, active fractions eluted from the DEAE-Sepharose column were dialyzed against 10 mM sodium phosphate buffer overnight to exchange the Tris-HCl buffer prior to loading the hydroxyapatite column, TRM was inactivated. These results indicate that TRM may be unstable at low concentrations of sodium phosphate buffer If the active fractions were allowed to remain at 4°C while protein and activity assays were being done and then pooled to load the hydroxyapatite column, the majority of TRM would be inactivated. A possible reason for the instability of this enzyme at this point in the purification procedure is the removal of one of the substrates, tRNAs. The presence of substrates stabilizes enzyme activity because they protect the active site from being modified and protect the protein conformation (Scopes, 1987). Transfer RNAs are removed during fractionation on DEAE-Sepharose (Kuchino and Nishimura, 1970) thereby possibly reducing the stability of TRM Assaying for TRM activity in the absence of added tRNAs will determine what role the tRNAs play in the stability of TRM activity and will determine whether tRNAs were removed by the DEAE-Sepharose column.

DEAE-Sepharose chromatography was useful in the purification of TRM Even though the increase in specific activity is only 2.8 - fold (Table 6), enrichment is obtained with this column because the total amount of protein

decreases from 6 g to 1.1 g. The high flow rate and the volume of resin allow lots of protein to be fractionated quickly. Also, all of the protein recovered from ammonium sulfate fractionation of 1 kg of lupin seeds could easily be loaded onto this column. Most importantly, if the activity recovered in the flow-through of this column is a separate TRM enzyme, then this column removes this enzyme from the TRM enzyme that I purified.

Only 30 % of TRM activity is recovered from the DEAE-Sepharose column. This low recovery was due to the approximately 50 % of TRM activity which did not bind to the resin and possibly to the instability of TRM in the absence of its tRNA substrate.

2. HYDROXYAPATITE CHROMATOGRAPHY

Hydroxyapatite chromatography is used in protein purification even though the mechanism of protein binding and elution has not yet been fully understood (Gorbunoff, 1985).

TRM was bound strongly to the hydroxyapatite column and could be eluted only with 400 - 500 mM sodium phosphate (pH 6.5) buffer (Figure 8). All of the recovered TRM activity was found in fractions 45 - 60, no TRM activity eluted prior to 400 mM sodium phosphate and there was no TRM activity in the flow through. However, we did not determine if all of the TRM activity loaded onto the hydroxyapatite column was eluted at 500 mM sodium phosphate because sodium phosphate was insoluble at 4°C and formed crystals in the

buffer and in the column when the concentration was increased past 500 mM. These crystals damaged the resin and affected the flow rate of the column. In order to avoid crystallization in the resin, the column had to be washed with dH_2O immediately after the sodium phosphate gradient was finished to remove residual sodium phosphate buffer However, extending the 500 mM sodium phosphate wash after the gradient was finished resulted in eluting more TRM activity from the column.

Initially, when a smaller hydroxyapatite column was used, a 10 - 400 mM or a 100 - 400 mM sodium phosphate (pH 6.5) linear gradient was used to elute TRM activity (data not shown). However, the recovered TRM activity was low. Extending the upper limit of the linear gradient from 400 mM to 500 mM sodium phosphate led to an increase in the amount of recovered TRM activity The low recoveries of TRM activity with 400 mM sodium phosphate was because some of the TRM activity was still bound to the column and could only be eluted with 500 mM sodium phosphate.

The protein and the TRM activity peaks were reproducible between hydroxyapatite columns. Protein always eluted as two peaks with the 10 - 500 mM sodium phosphate (pH 6.5) linear gradient (Figure 8) The first peak was sharp, eluting at approximately 80 mM sodium phosphate (pH 6 5) and the second was broader, eluting at approximately 300 mM sodium phosphate (pH 6.5). Active TRM was always localized at the end of the second protein peak

The optimum range and the volume of the sodium phosphate linear

gradient were determined. Taking the volume of the resin, the flow rate and the steepness of the gradient into account, a volume of 500 mls with a range of 10 - 500 mM was determined to be best. A smaller gradient volume did not yield good separation of TRM from other contaminating proteins and while a larger volume may have diluted TRM too much. Of the 60 - 70 fractions usually collected from the hydroxyapatite column, after addition of the gradient, TRM activity was found in approximately 15 of them (fractions #45 - 60).

Hydroxyapatite was selective for purifying TRM because the major protein peaks were well-separated from the TRM activity peak (Figure 8). Only 50 mg of protein was recovered from the 1 1 g that was loaded and a 39 - fold enrichment in specific activity was obtained (Table 6). Only 60 % of apparent TRM activity was recovered, however, this low recovery could be due to the presence of 500 mM sodium phosphate during the TRM activity assays or to the pH of the sodium phosphate buffer (pH 6.5). I have shown previously the TRM activity increases as pH increases from 7 5 to 8. Kwong (1975) has also shown maximum methylation of guanosine residues at pH 8.5.

3. TRANSFER RNA-AFFINITY CHROMATOGRAPHY

Since the tRNA-affinity column is designed to be specific for tRNA binding proteins it is not surprising that the majority of proteins were not bound to the column (Figure 9) Some proteins were not tightly bound to the tRNA affinity column and could be eluted from the column when the wash buffer was changed

from sodium phosphate to Tris-HCl. At times, some TRM was eluted here (data not shown) however, most of the TRM activity was tightly bound to the column and could only be eluted with 250 mM NaCl (Figure 9).

When the active fractions eluted from the tRNA-affinity column were analyzed with SDS-polyacrylamide gel electrophoresis these fractions showed many proteins (Figure 12). Given the large population of tRNA modifying enzymes in eukaryotic cells, as well as the other tRNA-binding proteins (processing enzymes, etc.) one might expect many tRNA-binding proteins to cofractionate with TRM even after ammonium sulfate fractionation, DEAE-Sepharose and hydroxyapatite chromatography. This column was also used in the purification of lupin tRNA nucleotidyltransferase and many proteins were also observed in the early fractions eluted with 200 mM NaCl (Shanmugam, 1994). In order to reduce the number of proteins seen in these fractions, a number of different elution methods were tried However, none of these methods showed any significant reduction in the number of bands in the active fractions nor an improvement in the recovered TRM activity

Transfer RNA molecules were bound to CNBr-activated Sepharose-4B resin. However, the coupling of the tRNA molecule to the Sepharose resin was not specific. Any exposed or accessible amino group on the tRNA molecule would be reactive towards CNBr This means that the orientation of the bound tRNAs was different and could not be controlled TRM is a tRNA-binding protein, however, it only methylates tRNAs at G_{26} (Edqvist *et al.*, 1992),

therefore, if a tRNA molecule is bound to the resin in such a way that G_{26} is not exposed, or if the tRNA is attached to the resin via an amino group on G_{26}, then TRM may not bind as strongly or at all. Total wheat germ tRNAs were used as the ligand on this column. It is possible that these tRNA are already fully or partially methylated at G_{26} and that some of these tRNAs contain A,C or U at position 26 instead of G. The cases where some TRM was eluted after switching the buffer, could be explained by either of these possibilities which may result in loose binding of TRM to tRNA.

The recovery of TRM activity from tRNA-affinity chromatography was 50 %. This low recovery could be due to the loss of some activity during the 50 mM Tris-HCl washing step and because this protein was dialyzed prior to the TRM activity assays to reduce the salt concentration. The tRNA-affinity column also was selective for TRM because only 3 mg of protein was recovered from the 49 mg that was loaded and a 394 - fold increase in specific activity was achieved.

4. ADENOSINE-AFFINITY CHROMATOGRAPHY

Adenosine-agarose-affinity chromatography was chosen to follow tRNA affinity chromatography because TRM should also show affinity for bound adenosine residues because adenosine is in S-adenosylmethionine, a substrate for this enzyme.

When approximately 3 mg of active protein was loaded onto the adenosine-agarose-affinity column, most of the protein and TRM activity was

collected in the flow-through (Figure 10). In order to determine whether or not the TRM activity collected in the flow through was due to the capacity of the column being surpassed, a smaller amount of this protein was reloaded onto the column. These results (Figure 11) show that by decreasing the amount of protein loaded onto the column, no TRM activity was collected in the flow through.

There was no apparent TRM activity eluted with 1mM SAM in TME buffer containing 200 mM NaCl. This may be due to the presence of 1 mM SAM in the assay buffer. This is a 91 - fold excess as compared to the level of SAM normally used in the TRM activity assay. This unlabelled SAM was probably competing for incorporation with [^{3}H]-SAM during the TRM activity assays. Dialysis in TME buffer for 4 hours was used to eliminate the 1 mM SAM. However, no active TRM was obtained possibly because it was inactivated during dialysis.

The adenosine-affinity column showed over 6000 - fold enrichment of TRM activity compared to crude lupin protein. The recovery of TRM activity was quite low, only 3 % of the activity loaded onto this column and only 0.3 % overall because most of this activity did not bind to the column (Figure 10) By using an adenosine-affinity column with a larger capacity, a higher yield of recovered TRM activity should be obtained.

E. SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-Polyacrylamide gel electrophoresis was used to verify the progress in the purification of TRM. The number of different proteins in the active fractions decreases greatly over the course of the purification (Figure 12). There are only a few different proteins present in the most purified, active fraction that is eluted from the adenosine-agarose-affinity column but activity cannot be correlated specifically with any of these bands. There is one band in the fraction eluted with 1 mM SAM from the adenosine-affinity column which corresponds to one of the bands in the active fraction but this fraction is not active. It is interesting that the proteins shared between these two fractions have an apparent molecular weight of 66 kDa which is the approximate molecular weight of the yeast *TRM1* protein (Ellis *et al.*, 1986).

F. WESTERN ANALYSIS

The results from the Western blot (Figure 13) showed that the yeast *TRM1* antibody cross-reacted with a protein at approximately 66 kDa in the most purified fractions eluted from the adenosine-affinity column. This antibody also cross-reacted with other proteins in the less pure fractions. However, in lanes 3, 4 and 5 which contain the same amount of crude, ammonium sulfate fractionated and DEAE-Sepharose purified protein, an increase in the intensity of the band at approximately 66 kDa is seen to parallel the increase in the purity of TRM. These data suggest that the single band in lane 9 that cross reacts with the

TRM1 antibody represents the lupin TRM enzyme.

G. PURIFICATION OF N^2-METHYLGUANOSINE$_{26}$-SPECIFIC tRNA METHYLTRANSFERASE

Over 6000 - fold enrichment of TRM activity was achieved after ammonium sulfate fractionation, DEAE-Sepharose, hydroxyapatite, tRNA-affinity and adenosine-affinity chromatography (Table 6). This purification procedure results in a single band on an SDS-PAGE gel which cross reacts with the yeast *TRM1* antibody. However, the final yield of TRM activity was low (0.3 %), therefore, more protein must be purified to give sufficient amounts of protein for further study.

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