PART ONE:
THE MINOR FORM OF HUMAN 5.8S RIBOSOMAL RNA

PART TWO:
PURIFICATION AND CHARACTERIZATION OF A RIBOSE TRANS METHYLASE FROM EURHICH ASCITES CELLS

by
David Smith

Dissertation submitted to the faculty of the
Virginia Polytechnic Institute
and State University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy
in Biochemistry and Nutrition

Approved:

----------------------------------
T. O. Sitz, Chairman

----------------------------------

B.M. Anderson               T.W. Kennan

----------------------------------

J.L. Johnson               J.E. Wiktorowicz

November 18, 1986
Blacksburg, Virginia
PART ONE: THE MINOR FORM OF HUMAN 5.8S rRNA
by David Smith
Committee Chairman: Thomas O. Sitz
Biochemistry
(Abstract)

An elongated form of 5.8S rRNA has been found in a wide range of eukaryotes from yeast to rodents. This minor form of 5.8S rRNA is about six nucleotides longer than the major form and composes from 10% to 30% of the total 5.8S rRNA found in yeast and rodents respectively. The minor form of 5.8S rRNA (pCCGAUA-) found in mice and rats may be generated by the formation of a secondary cleavage site caused by heterogeneity in the rRNA genes. The insertion of an adenylic acid residue in the precursor rRNA generates this additional cleavage site, i.e. -ACGA- or -ACCGA- for the major and minor forms respectively.

There is also heterogeneity with respect to the degree of methylation in rodent 5.8S rRNA. The conformation of the two chain length isomers is influenced by 2'-O-methylation of the uridylic acid residue at position 14, i.e. the most compact conformation is not ribose methylated in that position. The molecules which are methylated
in the 14th position cannot adopt the most compact conformation.

In the present study I have discovered a minor form of 5.8S rRNA in human placenta and I have determined its sequence; it differs from the major form of human 5.8S rRNA in having an additional sequence (CUCGUA) on the 5'-terminus. The sequence of the major rodent 5.8S rRNA is completely conserved in the major human 5.8S rRNA but the elongation on the 5'-ends of the minor 5.8S rRNAs from the two species are only 50% conserved. Human minor 5.8S rRNA was completely methylated at the uridylic acid residue at 14 making it the first 5.8S rRNA found to be completely methylated.
PART TWO: PURIFICATION AND CHARACTERIZATION
OF A RIBOSE TRANSMETHYLASE
FROM EHRlich ASCITES CELLS

by David Smith
Committee Chairman: Thomas O Sitz
Biochemistry

(Abstract)

A filter binding assay was developed for measuring ribose transmethylase activity in cell extracts and was used to quantify ribose and base transmethylase in Ehrlich ascites cells and normal mouse liver. Ribose and base transmethylase activities were elevated two-fold in Ehrlich ascites cells compared to normal mouse liver when methyl-deficient mouse tRNA was used as substrate but base transmethylase activity was elevated ten-fold in Ehrlich ascites cells when E. coli tRNA was used as substrate. E. coli tRNA did not serve as a methyl acceptor for ribose transmethylases.

The ribose transmethylase was purified 910-fold from Ehrlich ascites cell extracts and complete elimination of base transmethylase was achieved in one experiment. This purified ribose transmethylase was found to have an apparent
$K_{m_{TRNA}}$ of 20uM tRNA and an apparent $K_{m_{SAM}}$ of 12.8uM SAM. The apparent molecular weight of the ribose transmethylase, as determined by gel filtration chromatography, was 240,000 daltons. SDS-PAGE of the purified ribose transmethylase showed a predominant protein band of approximately 60,000 daltons.
ACKNOWLEDGEMENTS

I would like to express my gratitude to Thomas O. Sitz for the opportunity to work in his lab and benefit from his experience. I acknowledge also the members of my research committee, Bruce Anderson, Tom Kennan, John Johnson and John Wiktorowicz for their guidance and support. I thank my friends for their support and for the "good times". Finally, I thank my family for their love and patience.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS ........................................ i
TABLE OF CONTENTS ....................................... ii
LIST OF FIGURES ......................................... iii
LIST OF TABLES .......................................... vi

## PART ONE: THE MINOR FORM OF HUMAN 5.8S RIBOSOMAL RNA

- Literature Review ..................................... 1
- Experimental .......................................... 11
- Results .............................................. 19
- Discussion ........................................... 35
- References ........................................... 38

## PART TWO: PURIFICATION AND CHARACTERIZATION OF A RIBOSE TRANSMETHYLASE FROM EHRlich ASCITES CELLS

- Literature Review ..................................... 41
- Experimental .......................................... 49
- Results .............................................. 65
- Discussion ........................................... 126
- References ........................................... 139
- Vita .................................................. 143

vii
LIST OF FIGURES

figure number                  page

PART ONE:

1. Processing Scheme for rRNA........... 3
2. The "Burp Gun" Model for the Secondary Structure of 5.8S rRNA........... 6
3. Purification of the Minor 5'-\(^{32}\)P-5.8S rRNA from Human Placenta on Polyacrylamide Gels. ........................................ 21
4. Purification of the 5'-\(^{32}\)P-5.8S rRNA Isomers on Polyacrylamide Gels........... 23
5. Sequence Analysis of Human Minor 5.8S rRNA ........................................ 26
6. 5'-Terminal Analysis of Human Minor 5.8S rRNA ........................................ 29
7. Partial Base Hydrolysis of the Minor Forms of Human 5.8S rRNA................... 31
8. Sequencing Gel of the Major 5.8S rRNA (Conformer A).......................... 34

PART TWO:

1. Assays of In vitro Transmethylase Activity ........................................... 55
2. Total Transmethylase Activity of Ehrlich Ascites and Mouse Liver Extracts on E. coli tRNA ........................................... 71
3. Total Transmethylase Activity of Ehrlich Ascites and Mouse Liver Extracts on Methyl Deficient Mouse tRNA............73

4. DEAE Sephadex Analysis of Transmethylase Activities in Ehrlich Ascites Homogenates Using E. coli tRNA as Substrate.. 75

5. DEAE-Sephadex Analysis of Transmethylase Activities in Ehrlich Ascites Homogenates Using Methyl Deficient Mouse tRNA as Substrate.......................76

6. Determination of In Vitro Ribose Methylation of Methyl Deficient Mouse ³H-tRNA by Filter Binding Assays.....................80

7. Enzymatic Digestions for Analysis of ³H-tRNA ........................................83

8. DEAE-Sephadex Chromatography for Determining the Size of Alkaline Resistant RNA Fragments. ........................................89

9. Sephacryl S-300 Gel Filtration Chromatography of Transmethylase.................93

10. Matrex Gel Green A Chromatography of Transmethylase........................................97

11. DEAE-Sephadex Analysis of Transmethylase Activity Eluted from a Matrex Gel Green A Column ..................99

12. DEAE-Sephadex Analysis of Ribose and Base
Transmethylase Activity in Partially Purified Ribose Transmethylase Using Methyl Deficient Mouse tRNA as Substrate.............. 102
13. The Effect of pH on the Ribose Transmethylase Activity....................... 105
14. The Effect of KCl Concentration on the Ribose Transmethylase Activity........... 107
15. Determination of the Apparent Molecular Weight of Ribose Transmethylase by Sephacryl S-300 Chromatography............................... 110
16. Determination of an Apparent $K_m^{tRNA}$ for the Ribose Transmethylase ............. 112
17. SDS-PAGE of Ribose Transmethylase Fractions ...................................... 115
18. Determination of an Apparent $K_m^{SAM}$ for the Ribose Transmethylase.............. 118
19. DEAE-Sephadex Analysis of Ribose and Base Transmethylase Activity in Partially Purified Ribose Transmethylase Using E. coli tRNA as Substrate................................. 122
20. Effect of "Aging" on the Ribose Transmethylase ...................................... 125
LIST OF TABLES

<table>
<thead>
<tr>
<th>table number</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Perchloric Acid Digestions</td>
<td>86</td>
</tr>
<tr>
<td>2. Purification Table</td>
<td>100</td>
</tr>
<tr>
<td>3. Nucleic Acid Substrate Studies</td>
<td>120</td>
</tr>
<tr>
<td>4. Characteristics of Some Transmethylases</td>
<td>137</td>
</tr>
</tbody>
</table>
LITERATURE REVIEW

In eukaryotic cells the initial rRNA transcript is a 45S precursor rRNA which is processed (Figure 1) by a series of enzymatic cleavages (1,2,3). This precursor has an 18S sequence near the 5'-end, a 5.8S sequence in the middle, and a 28S sequence near the 3'-end of the molecule. Internal transcribed spacer regions (ITS) located between the conserved rRNA sequences and terminal regions located at the 5' and 3'-ends are enzymatically cleaved during processing. The 5.8S rRNA is a component of the 60S ribosomal subunit, where it is hydrogen-bonded to the 28S rRNA. The mature ribosomes in eukaryotic cells contain a 60S subunit and a 40S subunit which are composed of RNA and associated proteins.

There are considerable data in support of this rRNA processing scheme but additional studies have revealed that it does not completely describe all rRNA processing. For example, three processing pathways have been discovered in mouse cells, one of which was previously thought to be unique to HeLa cells(4). In these experiments,
Figure 1
Processing Scheme for rRNA
nuclear RNAs were separated by agarose gel electrophoresis, transferred to diazo-paper, and hybridized to twelve different restriction fragments complementary to sections of the 45S pre-rRNA. Four new pre-RNAs were discovered, and the three new pathways were proposed to explain them, each pathway being unique in the order of the cleavages. This same study revealed a 12S precursor to 5.8S rRNA in mouse cells.

A "burp gun" model (figure 2) has been proposed for the secondary structure of the 5.8S rRNA(5) based on studies of 5.8S rRNA from yeast and higher eukaryotes. This model contains five base paired regions. The ends of the molecule fold together to produce the horizontal base paired regions. The vertical paired regions, known as hairpin loops, are formed by self complementary regions of the molecule. The researchers who proposed this model used partial U2 and T1 ribonuclease digests to generate RNA oligonucleotides which were then mapped by two-dimensional paper electrophoresis. When mild conditions were used in the enzyme digest, hydrolysis occurred mostly in the open, non-base paired regions of the molecule. These data indicated which sequences in the molecule were base paired
Figure 2
The "Burp Gun" Model for the Secondary Structure of Rat 5.8S rRNA
and which were not. Khan and Maden (6) conducted similar experiments using S1 nuclease, which cleaves predominantly in non-base paired regions of the molecule, and their data also supported the "burp gun" model. Although the "burp gun" model for the secondary structure of 5.8S rRNA is generally accepted there is also evidence for the variation of this basic structure occurring in different species of organisms. For example, rat 5.8S rRNA has greater electrophoretic mobility than yeast 5.8S rRNA on polyacrylamide gels (PAGE) suggesting that rat 5.8S rRNA has a more compact conformation. Turtle and chicken 5.8S rRNA have intermediate mobilities. There is an adenylic acid residue near the 5'-terminus in both turtle and chicken 5.8S rRNA. Human and rat 5.8S rRNA sequences have a guanylic acid residue in this position. This nucleotide substitution has been proposed to weaken base pairing, allowing the 5' and 3'-ends of the molecule to separate, producing a more open conformation (7).

5.8S has high propensity for RNA-RNA interactions, such as the formation of dimers (8) and the formation of a complex with 28S rRNA (1). These types of interactions are probably important to the functioning of 5.8S rRNA in the ribosome. The
function of 5.8S rRNA in the ribosome is unknown.

We have obtained data correlating the conformation of 5.8S rRNA and the 2'-O-ribose methylated uridylate in the 14th position(9). Generally ribose methylation occurs at the level of the 45S rRNA precursor in the nucleolus (10,11,12), but the 14th position modification in 5.8S rRNA is a late cytoplasmic event(13). Recently additional data were obtained suggesting that different transmethylases are responsible for the two methylations of 5.8S rRNA; 0.1uM neplanosin A (an adenosine analog) in the culture media of cultured normal rat kidney (NRK) cells selectively inhibits the cytoplasmic methylation, UmG, but had no effect on the nucleolar methylation, GmC, even at a concentration of 100uM(14).

The phenomenon of hypomethylation of nucleic acids in cancer cells is currently an area of intense interest. Randerath et al.(15) has sequenced the Morris hepatoma serine Ser₁-tRNA and compared it to its normal counterpart in the rat liver. The tumor Ser₁-tRNA lacks the ribose methylation of a guanylic acid in position 17 of the dihyrouridine loop. They also contend that the column chromatographic behavior of Ser₁-tRNA lacking this ribose methylation suggests a con-
formation change which might affect its function in translation. The 5.8S rRNA extracted from cancer tissue is hypomethylated at the 14th position UmG compared to 5.8S rRNA extracted from normal tissue(13) and this 2'-O-ribose methylation also affects the conformation of the molecule(9).

The exact function of RNA 2'-O-methylation is unknown, but nucleolar methylation is related to proper rRNA processing(16). When Hela cells are starved of methionine, a precursor to the methylating agent S-adenosylmethionine (SAM), no complete ribosomes are formed even though there is no inhibition of rRNA transcription. As a control, when Hela cells are starved of valine, which is not directly required for methylation, complete ribosomes are produced for many hours. Also certain bacteria use 2'-O-ribose methylation as a defense mechanism against antibiotics which act as inhibitors of protein synthesis(17). The bacterium *S. azureus* has a 2'-O-ribose methylated adenosine in its 23S rRNA, and is known to be resistant to thiostreptin, an inhibitor of protein synthesis which binds to the ribosome. A transmethylase, isolated from this organism, is able to methylate core ribosomal particles. Incorporation of methyl groups at the ribose
moiety of adenosine corresponds to resistance to thiostreptin binding.

An elongated form of 5.8S rRNA has been found in yeast(2) and rodents. In a previous study by the author(9), the elongated form of 5.8S rRNA in Ehrlich ascites mouse tumor and mouse liver was determined to have an additional (C)CGAUA- on the 5'-terminus. It was proposed that this elongated form is generated when the 5'-processing nuclease recognizes this -ACCGA-sequence as the 5'-processing site instead of the -ACGA- in the transcript of the major form. Both of these sequences are similar to a rRNA processing consensus proposed by Subrahmanyam et al.(18). Studies of the minor forms of 5.8S rRNA in other organisms may contribute to the understanding of rRNA processing. In the present study I have discovered a minor form of 5.8S rRNA in human placenta and determined its sequence and methylation pattern.
EXPERIMENTAL

1. Materials

Tris(hydroxymethyl)aminomethane (Tris), 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (Hepes), and dithiothreitol (DTT), were purchased from Sigma Chemical Company Chemical. T₄-infected E.coli RNA ligase, calf intestinal mucosa alkaline phosphatase, T₄-infected E.coli polynucleotide kinase and RNA sequencing enzymes were obtained from P-L Biochemicals Inc. Sequencing Gel Stands were purchased from Bethesda Research Laboratories, Inc. Human placenta was kindly supplied by Dr. Miguel Langebeck, Montgomery County Hospital. All other chemicals were reagent grade.

2. Isolation of RNA from Human Placenta

Approximately 20 g of frozen human placenta were cut into small pieces. The tissue was ground in a blender for 10 seconds, mixed with ten volumes of sodium dodecyl sulfate buffer (SDS buffer) (0.14M NaCl, 0.05M Na acetate, 0.3% w/v SDS, pH 5.1), and subsequently with an equal volume phe-
nol-cresol solution (75% phenol, 16% m-cresol, and 9% H₂O v/v, and 1g/liter 8-hydroxyquinoline). The mixture was heated to 55° with continuous stirring, chilled to room temperature in an ice bath and stirred for an additional 30 minutes at room temperature. The phases were then separated by centrifugation at 3,000xg(5) and the aqueous layer was collected. The RNA was precipitated with two volumes of 95% ethanol (2% potassium acetate) at -20° for 8 hours, pelleted by centrifugation (3,000xg for one hour), and finally washed with 95% ethanol. The RNA pellet was dissolved in autoclaved water and the concentration was determined by absorbance at 260nm (E₁₅= 200).

3. Polyacrylamide Gel Electrophoresis (PAGE) of RNA.

PAGE was used to purify 5.8S rRNA. A Tris borate buffer [0.088M Boric acid, 2.0mM ethylenediamine-tetraacetic acid (EDTA), 0.086M tris(hydroxymethyl)aminomethane (Tris), pH 8.3] was used to make acrylamide solutions and as the electrophoresis buffer (19). Preparative PAGE [10% acrylamide (1:40 crosslinking) with 7M urea] was used to purify 5.8S rRNA from whole cell RNA and PAGE on 15% acrylamide gel (1:40 crosslinking) was
used for additional purification of 5.8S rRNA. The RNA in the gels was stained with Methylene blue (0.1% w/v) in 5% acetic acid. Autoradiography was used to visualize radioactive RNA. The bands were cut from the gel and the radioactivity was quantified by Cerenkov counting. RNA was extracted from gel slices by homogenizing the gel in a salt buffer [0.15M NaCl, 0.001M morpholino-propane sulfonic acid (MOPS), 0.001M EDTA, pH 7.2]. The homogenate was mixed for 20 minutes, frozen, then thawed; this procedure was repeated three times. The acrylamide was pelleted by centrifugation then the supernate was removed and mixed with two volumes of 95% ethanol (2% potassium acetate). The sample was kept at -20°C for several hours and then the RNA was pelleted by centrifugation at 2,000xg for 45 minutes. The pellet was washed with 95% ethanol, allowed to dry, and then suspended in autoclaved water. The concentration was determined by absorbance at 260nm (E₁%=200).

4. **Enzymatic Labeling of the 3'-terminus of 5.8S rRNA**

A sample of purified 5.8S rRNA and 100uCi 5'[^32P]-cytidine bisphosphate were dried in a 1.5
ml microcentrifuge tube. Two microliters of buffer [0.5M 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (Hepes), 0.15M MgCl₂ and 33mM dithiothreitol (DTT)], 2ul of dimethyl sulfoxide, 2ul of bovine serum albumin (0.1g/ml, fraction V BSA heated at 80° for 3 minutes), 2ul of 0.26mM ATP and 4ul of stock RNA ligase (2000 units/ml) were added. The mixture was incubated overnight at 4°(20), diluted with an equal amount of autoclaved water and formamide, and mixed. Five microliters of formamide-dye mix (0.03% xylene cyanole, 0.08% bromphenol blue) was added. The sample was heated to 90° for one minute and the 5.8S rRNA was purified by PAGE. The radioactive RNA was located by autoradiography and extracted from the gel by homogenization as described above.

5. Enzymatic Labeling of the 5' Terminus of 5.8S rRNA

The RNA sample was first treated with alkaline phosphatase, which hydrolyzes terminal phosphates, and then reacted with polynucleotide kinase plus ATP, which transfers a γ-phosphate from ATP to the 5'-terminus of RNA. Purified 5.8S rRNA was dried in a microcentrifuge tube, dis-
solved in 4ul of alkaline phosphatase solution (3 units/ml) plus 4ul water, and incubated at 37° for one hour. The sample was extracted with 50ul water and 50ul phenol mixture, centrifuged at 15,500xg for 5 minutes, and the aqueous phase removed. The phenolic phase was washed with 25ul water and the aqueous phases were combined and washed 3 times with diethyl ether. The ether was removed and the RNA was precipitated, at -20°C with three volumes of a 95% ethanol (2% potassium acetate) solution. The RNA was pelleted at 15,500xg for 5 minutes, washed with 95% ethanol, and dried. The pellet was dissolved in 10ul of buffer (10mM Tris HCl, pH 7.4, 1mM spermidine, 0.1mM EDTA) and heated at 50° for 3 minutes, then chilled on ice. This solution was transferred into a microcentrifuge tube containing 100uCi of dried γ-[³²P]-ATP followed by the addition of 0.5ul stock polynucleotide kinase solution (2000 units/ml), 2ul kinase buffer (0.5M Tris HCl, pH 9.0, 100mM MgCl₂, 50mM DTT) and was incubated at 37° for 25 minutes(21). The sample was heated to 90° for 30 seconds, chilled on ice, and then the 5.8S rRNA was isolated by PAGE.
6. The Enzymatic Sequencing Procedure

RNA labeled on one terminus with $^{32}$P was hydrolyzed with base specific enzymes and the resulting fragments separated by high resolution PAGE(22). Base specific ribonuclease enzymes used in the procedure were: T2 RNase for (G), U2 RNase for (A), Phy M RNase for (A and U) and B. cereus RNase for (C and U). Uracil for example, is indicated by a cleavage in the Phy M and B.c. lanes. The conditions below are those used to produce the sequencing gel shown in figure 5, however, conditions were adjusted in other experiments depending on the activity of the enzymes at that time.

Five samples of purified 5'-terminal labeled 5.8S rRNA (5,000-10,000 cpm) were dried in separate microcentrifuge tubes. Alkaline hydrolysis is performed on the fifth sample which was electrophoresed together with the others hydrolysis products and serves as a reference, since it produces a band for each position in the sequence. All samples and solutions were kept on ice prior to the incubations. Three microliters of buffer I (33mM sodium citrate, pH 5.0, 1.7mM EDTA, 0.04% xylene cyanole, 0.08% bromophenol blue, 1mg/ml carrier RNA, and 7M urea) were added to the RNA
sample for Tl and Phy M RNase digests. Three microliters of buffer II (same as buffer I, except at pH 3.5) were added to the sample for the U2 RNase digest, and 3ul of buffer III (same as buffer I, except containing no dyes or urea) were added to the sample for the B. cereus RNase digest. Diluted enzymes were then prepared in separate microcentrifuge tubes using autoclaved water. One microliter samples (1 unit) of the stock RNases Tl, B. cereus, and U2 RNases (all reconstituted according to the manufactures specifications) were diluted to 40ul, 10ul, and 7ul, respectively, gently mixed in vortexing, and 1ul of each diluted enzyme solution was added to the RNA samples containing buffer. One microliter carbonate solution (50mM NaHCO₃, 1mM EDTA) and 4ul formamide-dye solution was added to the RNA sample for alkaline hydrolysis. The samples were mixed on a Vortex. The Tl digest was incubated for 12 minutes, while the other three digests were incubated for 16 minutes, all at 55°. The alkaline hydrolyzed sample was heated for 12 minutes at 90°. All the samples were chilled on ice and 2ul of a formamide-dye mixture was added to each. The samples were loaded for PAGE without prior heating. Electrophoresis was on 12% poly-
acrylamide gels (1:19 crosslinking) containing 8.5M urea at 1,500 V (as described in section 3).

7. Terminal Nucleotide Analysis of Minor 5.8S rRNA

Samples of each of the four chain length isomers of human minor 5.8S rRNA labeled at their 5' termini were digested with 20μl of 0.3N NaOH for 16 hours at 37°. The digested samples were applied to Whatman 3MM paper and electrophoresis was performed (5% acetic acid, pH 3.5 buffer) at 1500 volts (23). The separated digestion products were detected by autoradiography.
RESULTS

8. Purification and In Vitro

5'[32]P-Phosphorylation

of Minor 5.8S rRNA From Human Placenta

Total RNA was extracted from human placenta, separated by PAGE (15% acrylamide, 7M urea) and stained with methylene blue. The 5.8S rRNA migrated as two poorly resolved bands, an intensely stained band and a slower migrating, lightly stained band. The RNA in the slower migrating band was recovered as previously described. The recovered RNA was labeled at the 5'-terminus by incubation with [32]P-ATP and polynucleotid kinase. The mixture was electrophoresed on a 15% polyacrylamide gel with 7M urea. The radioactive RNA bands were visualized by autoradiography. The radioactive 5.8S rRNA migrated as two bands of equal intensity. The faster migrating band was the major 5.8S rRNA, present as a contaminant, and the slower migrating band was the minor 5.8S rRNA (figure 3). The minor 5.8S rRNA was extracted from the gel and further purified by two more sequential electrophoretic separations on native 15% polyacrylamide gel. The resulting autoradiograph (figure 4)
The 5.8S rRNA purified on a 7M urea 15% polyacrylamide preparative gel was extracted from the gel and labeled on the 5'-terminus by incubation with $^{32}$P-ATP and polynucleotide kinase and then the incubation mixture was electrophoresed on a 15% polyacrylamide gel with 7M urea and the radioactive RNA bands were visualized by autoradiography. The left two lanes contain samples enriched in minor 5.8S rRNA after purification on the preparative gel. The right two lanes contain RNA extracted from the region of the preparative gel just above the band representing the major 5.8S rRNA. This region of the preparative gels often contains a small amount of the minor 5.8S rRNA.
Figure 4
Purification of $5'-{\text{32P}}-5.8S$ rRNA Isomers on Polyacrylamide Gel

$5'-{\text{32P}}-\text{Minor 5.8S rRNA}$ was electophoresed on native 15% polyacrylamide gels. The four conformational isomers were extracted from the gel and applied to separate lanes of a second native gel (above) to achieve additional purification of the four isomers. The lanes (left to right) were isomer "a" (six lanes), isomer "b" (three lanes), isomer "c" (three lanes) and isomer "d" (two lanes).
shows four distinct bands which represent minor forms of human 5.8S rRNA differing in length by a single nucleotide (see below).

9. Sequence Analysis of Human Minor 5.8S rRNA

The four RNA's isolated by the series of electrophoretic separations described above were sequenced by the enzymatic degradation-rapid sequencing gel technique (section 6). The longest of the four had an additional (pCUCGUA-) at its 5'-terminus and the other three RNA's were sequentially one nucleotide shorter on the 5'-terminus (figure 5). Similar experiments conducted with 3'-labeled 5.8S rRNA showed that there are no differences in the major and minor forms on the 3'-termini.

10. The 5'-Terminal Analysis of Human 5.8S rRNA

The four minor human 5.8S rRNA's appeared to be identical in sequence except for the differences in chain length; therefore, it was possible to confirm the sequence of the last four residues on the 5'-terminus of the longest molecule by identifying its 5'-terminal nucleotide and the 5'-terminal nucleotides of each successively
figure 5
Sequence Analysis of Human Minor 5.8S rRNAs

The four 5'-32P-RNA's, isolated from the polyacrylamide gel shown in figure 4, were sequenced by the enzymatic degradation-rapid sequencing gel technique.
shorter RNA. The 5'-terminal nucleotides of the four minor 5.8S rRNA's were pCp, pUp, pCp and pGp in order of the longest to the shortest RNA (figure 6). These terminal analysis supported the sequence obtained from the rapid sequencing gels. A general problem with the enzymatic degradation-rapid sequencing gel technique is that often terminal nucleotides are not cleaved by the base specific enzymes, i.e., B. cereus RNase does not cleave terminal cytidylic acid residues(24).

11. The 2'-O-Ribose Methylation of the 14th Uridylic Acid in Human 5.8S rRNA

I have found that the 14th uridylic acid was completely 2'-O-ribose methylated in the major and minor forms of human 5.8S rRNA. Rapid sequencing gels of the minor 5.8S rRNA from human placenta did not have a band corresponding to base hydrolysis at residue 14 (figure 7) because 2'-O-methylated residues are resistant to alkaline hydrolysis. Similar experiments with the major 5.8S rRNA(data not shown) also demonstrated complete methylation.

12. Correction of the Previously Published Rat 5.8S rRNA Sequence
Figure 6
The 5'-Terminal Analysis of Human 5.8S rRNA

The 5'-32P labeled samples of each of the four conformational isomers of human minor 5.8S rRNA were digested with 20 ul of 0.3N NaOH for 16 hours and the samples were applied to Whatman 3mm paper and electrophoresed at 2,000 volts in 5% acetic acid, pH 3.5 buffer. The separated digestion products were viewed by autoradiography.
Figure 7
Partial Base Hydrolysis of the
Minor Forms of Human 5.8S rRNA

High resolution sequencing gel of partial base hydro-
lysis of the minor form of 5.8S rRNA. The "break" in the
ladder pattern was the result of complete 2'-O-ribose
methylation at UmG.
A dinucleotide (GC) in the originally reported rat 5.8S rRNA sequence (5) was not found in more recent studies where the rDNA was sequenced (18). The original study used the classical RNA sequencing methods (23) and reported a (GC) dinucleotide in the 51st position. This dinucleotide was in a region of the molecule which was difficult to sequence by the newer rapid sequencing techniques because there was compression in this region of the gel and sequencing from the 5'-terminus was precluded by the 5'-terminal heterogeneity of rat 5.8S rRNA. However, I separated the conformational isomers of 5'-labeled 5.8S rRNA on native 15% polyacrylamide gels to obtain the three isomers. The fastest migrating isomer (isomer "A"), which had a homogeneous 5'-terminus, was sequenced (figure 8). The sequence was found to be -AGCUA- instead of the originally reported -AGCGCUA-. The "ladder" in this region was easy to read with no signs of compression; therefore, the sequence of rat 5.8S rRNA now corresponds to the reported rDNA sequence. Similar studies (data not shown) indicate that human 5.8S rRNA also lacks this GC dinucleotide.
The conformational isomers of 5'-labeled rat 5.8S rRNA were separated on native 15% polyacrylamide gel to obtain the three conformational isomers. Conformer "A", which has a homogeneous 5'-terminus, was sequenced by the enzymatic degradation rapid sequencing gel technique.
There have been previous reports of an elongated form of 5.8S rRNA in a eukaryote, yeast, and in rodents\(^9\)\(^{(9)}\)\(^{(25)}\). The minor, i.e. elongated, 5.8S rRNA from mouse Ehrlich ascites cells and rat liver has been sequenced\(^9\) and found to have a \((pCCGAUA)\) sequence on its 5'-terminus which is absent in the major form. In the present study of the minor 5.8S rRNA from human placenta I have determined that there was a series of minor human 5.8S rRNAs, which differ in chain length and that the longest molecule had a \((pCUCCGUA)\) sequence on its 5'-terminus that was absent in the major human 5.8S rRNA. The major 5.8S rRNA sequence was highly conserved between rodent and human but the extra six nucleotides in minor sequences vary by 50%. It was not clear how the human minor 5.8S rRNA was generated, but it may have resulted from gene heterogeneity which allows for an alternative cleavage site during processing as was proposed for the rodent minor 5.8S rRNA\(^9\). Labeling kinetics\(^9\) suggested that the minor 5.8S rRNAs were not precursors to the major 5.8S rRNAs but were instead stable forms found in the ribosome. The minor forms represent 25-30% of the total
human 5.8S rRNA and exhibits greater chain length variation, one to six nucleotides, than the two rodent minor 5.8S rRNAs which differ in chain length by one nucleotide. The chain length variation of the human minor forms may result from an exonuclease activity leaves a 5'-phosphate after an initial endonuclease cleavage which generates the longest molecule. The function of these minor forms of 5.8S rRNA remains obscure, but the sequence of the added nucleotides may not be as important as the chain length of the elongation, since it was not as highly conserved as the major 5.8S rRNA sequence. The rodent minor 5.8S rRNA has been shown to form a more stable complex with 28S rRNA than does the major form(9) so it may play an important role in the structure and function of the ribosome. Rodent rDNA sequences have been published which correspond to the major and the minor forms of 5.8S rRNA(18)(26); therefore there appears to be heterogeneity in the rodent rDNA genes. The major 5.8S rRNA transcript in rodents has an -ACGA-sequence and is cleaved between the A and C residues to generate the major 5.8S rRNA. The minor 5.8S rRNA transcript in rodents has an adenylic acid inserted generating an -ACCGA- sequence which apparently acts as
an alternative cleavage site upstream from the normal cleavage site. When cleavage occurs between the A and C residues at this alternative site, the minor 5.8S rRNA is generated. Until the DNA from a number of clones is sequenced on the 5' side of human 5.8S rRNA it will not be possible to determine the mechanism by which the minor form of 5.8S rRNA is generated in human cells.
REFERENCES


25. Bowman, L.H., Goldman, W., Goldberg, G., Herbert, B.

PART TWO:
PURIFICATION AND CHARACTERIZATION
OF A RIBOSE TRANSMETHYLASE
FROM EHRLICH ASCITES CELLS
The methylated components of RNA fall into two general categories: nucleotides with methylated bases and nucleotides with methylated ribose. The bases are methylated on various positions but the only position available for methylation on the ribose is the 2'-O-position. There have been a number of studies (see review, reference 11) of the cellular location of the RNA methylations in vivo but many of the details remain obscure. The early rRNA methylation predominantly occurs in the nucleolous where only conserved ribosomal sequences are methylated, i.e. spacer sequences are not methylated (1). Most of the methyl groups are incorporated in the 45S precursor RNA shortly after transcription but a few groups are added later during ribosomal maturation (1), and still others are late modifications of mature rRNA in the cytosol (2). The biological functions of rRNA methylation remain obscure, although there is a general correlation between inhibition of early rRNA methylation and inhibition rRNA processing. For example, culture cells starved of methionine (3), a precursor of S-adenosylmethionine (SAM), did not synthesize 60S
ribosomal subunits, instead the cells accumulated an undermethylated 32S precursor. In other experiments(4), cycloleucine, a specific inhibitor of SAM biosynthesis, was administered to culture cells resulting in a 95% decrease in rRNA methylation and a decreased rate of ribosome synthesis. One interpretation of these data is that normal RNA methylation is necessary for RNA processing, but if methylation does function as a part of the RNA processing mechanism, its exact role is unclear.

Transfer RNA is also methylated but there is no generalization concerning the timing of these methylations during tRNA maturation; methylation occurs before, during and after trimming of precursor tRNA(5). It is currently believed that most eukaryotic tRNA transmethylases are localized in the cytosol and in the mitochondria(5) [although tRNA transmethylases have been detected in the nucleus of mouse L cells and Xenopus oocytes(6)]. Ribose methylation occurs at very specific sites in tRNA, usually in the loop regions such as the anticodon loop, the D-loop (contains dihydrouracil), and the T-loop (contains ribothymidylate). This finding suggests that methyl ribose may protect tRNA from
nucleases because the looped regions would be the most accessible regions on the molecule. Because ribose methylation may also affect tRNA conformation. Morris hepatoma ser₁-tRNA which lacks a specific late cytoplasmic ribose methylation was shown to have a different conformation than the methylated ser₁-tRNA from rat liver(7). Late cytoplasmic methylation has also been shown to effect the conformation of 5.8S rRNA which has a more open conformation if ribose methylated on the uridylic residue at the 14th position (8)(9).

The rRNA transmethylases and rRNA precursors from numerous tissues have been associated with the nucleolus(10) and it is suggested that, in the tissues studied, most rRNA transmethylases are nucleolar. There is also strong evidence that some rRNA methylations are late cytosolic events (2). The tRNA transmethylases and unmethylated tRNA precursors have been associated with the cytosol, but recent data argue that some tRNA transmethylases and tRNA precursors may leak from the nucleus during isolation(5,6) however the issue is still in debate. Another point of uncertainty relates to the ability of some crude tRNA transmethylase preparations to methylate rRNA,
but this phenomena may result from nonspecific tRNA transmethylase activity, rRNA specific activity leaked from nuclei, or cytosolic rRNA transmethylases (11).

Only a few RNA transmethylases (5) have been purified because of the inherent difficulties involved. One major difficulty has been in devising adequate assays for specific RNA transmethylases (5). An RNA transmethylase may be specific with regard to any number or all of the following criteria: nucleotide specificity, position specificity (various positions on the bases or the second hydroxyl on the ribose), sequence specificity, or substrate specificity (a particular RNA species). Analysis of the methylated components of RNA is usually by time consuming chromatographic or electrophoretic procedures, also it is expensive to obtain pure species of RNA to be used as substrate especially if the natural substrate is an unmethylated precursor RNA.

The study of tRNA transmethylase mixtures in crude extracts likewise has some inherent difficulties (5). If the assay employed detects a number of transmethylases in the extract without distinguishing among them, it is not possible to detect selective loss of certain activities as
the extract is stored or purified. Despite these difficulties, there have been several important studies on tRNA transmethylases in cell extracts or with partially purified enzymes. Rodeh(12) assayed RNA transmethylases in extracts of normal and regenerating liver using (\(^{14}\)C)-methyl-SAM and a variety of RNAs as substrates. The (\(^{14}\)C)-methylated components in RNA hydrolysates were analyzed by paper electrophoresis and five types of (\(^{14}\)C)-methyl bases were identified, suggesting the presence of five base specific activities. Total activity was maximized at pH 8.8 in the presence of 0.25M NH\(_4\)\(^+\), however no attempt was made to characterize the five individual activities because the techniques used to detect them were not practical for detailed enzyme studies.

Most of the soluble transmethylases purified have been prokaryotic tRNA transmethylases. However, a few eukaryotic tRNA transmethylases have been purified. In 1979 Nau(13) achieved a 350-fold purification of mouse plasmocytoma 1-adenine transmethylase using a combination of DEAE-cellulose chromatography and RNA Sepharose affinity chromatography. The purified enzyme had a broad pH optimum (pH7-pH9), and was stimulated
five fold by 0.15M NaCl(13). No tRNA ribose transmethylases have been purified to homogeneity but other types of ribose transmethylases have been at least partially purified(14). For example a nucleolar rRNA ribose transmethylase has been purified 90-fold(14).

Much of the current interest in tRNA transmethylases is related to reports that tRNA transmethylase activity is elevated in tumor cells. In 1983, Yanokura, et al.(15) compared several transmethylase activities in Ehrlich ascites tumor to the corresponding activities in normal mouse liver. They found that phospholipid, glycine, and guanidoacetate transmethylase activities are equivalent in tumor cell and normal cell extracts. However, tRNA transmethylase activity is elevated six-fold in the tumor cell extract. Experiments in several labs have demonstrated that administration of liver carcinogens, such as ethionine and dimethylaminoazobenzene, caused elevation of liver tRNA transmethylase activity(16,17). Transmethylase hyperactivity and, paradoxically, hypomethylated nucleic acids are a general phenomenon in tumor cells. The resolution of this paradox will be important in understanding the neoplastic state.
The *in vivo* ribose methylation of 5.8S rRNA has been studied previously in this lab and several observations made during those studies influenced the directions of the current project. One such observation that I found particularly intriguing is that the 5.8S rRNA extracted from cancer cells is hypomodified at the 14th position (UmG) compared to the 5.8S rRNA from normal tissue (18). The hypomethylation of 5.8S rRNA from cancer cells might be related to differences in the transmethylases of transformed and normal cells. This observation was the impetus for the current project, to study the ribose transmethylases in transformed and normal tissue. My initial efforts were directed toward the detection of an *in vitro* 5.8S rRNA-specific ribose transmethylase but these efforts were unsuccessful. I was able to demonstrate a tRNA-specific ribose transmethylase activity in crude extracts [this activity had also been reported by a previous worker in the laboratory(19)]. Therefore the focus of the project was directed toward tRNA ribose transmethylases, specifically the focus was on the purification of a ribose transmethylase activity in crude extracts of Ehrlich ascites cells so that the transmethylase could be
studied in a controlled environment.
EXPERIMENTAL

1. Materials

Morpholinopropane sulfonic acid (MOPS) was obtained from Calbiochem. [2(N-morpholino)ethane sulfonic acid] (MES), Tris (hydroxymethyl) aminomethane (TRIS) and dithiothreitol (DTT) were obtained from Sigma Chemical Company. L-ethionine was purchased from Sigma Chemical Company. Adenine was purchased from Pharmacia. Nonidet N-P40 was a gift from Dr. Thomas Kennan and is manufactured by Gallard Schlesinger. Phenol and Scintiverse E were obtained from Fisher Scientific. (³H)-SAM and (¹⁴C)-SAM were purchased from the NEN Research Products Division of E.I. du Pont. Nonradioactive SAM and E. coli tRNA were purchased from Boehringer-Mannheim. Diethylaminoethane (DEAE)-Sephadex was obtained from Sigma Chemical Company. Sephacryl S-300 was obtained from Pharmacia. Matrex Gel Green A was obtained from Amicon Corporation. ICR mice were obtained from Dominion Labs.
2. 100,000xg Extract from Ehrlich Ascites Cells and Mouse Liver

Ehrlich ascites tumor cells were grown in the peritoneal cavity of ICR mice(20) by injecting 4 \( \times 10^6 \) cells per animal. After one week, cells amounting to three to four milliliters packed volume were harvested. The tumor cells were suspended in hypotonic buffer[0.01M TRIS, 0.01M NaCl, 0.0015M MgCl₂, 0.001M DTT] to lyse the contaminating red blood cells and then pelleted at 200xg. This procedure was repeated until no additional hemolysis was detected in the suspended cells. The tumor cells were homogenized in 4.5ml of hypotonic buffer per milliliter packed cell volume using 60 strokes of a motor-driven teflon pestle(1,000rpm) and a glass Thomas type C homogenizer(21). The homogenate was centrifuged at 1,000xg for 10 minutes to pellet nuclei and cell debris, the supernate was centrifuged at 20,000xg for 20 minutes to remove mitochondria and lysosomes, and this supernate was centrifuged at 100,000xg for 2 hours to pellet microsomes. This 100,000g supernate(S100) was used as either a source of enzyme activity or tRNA. Endogenous RNA was removed from the supernate by adjusting the solution to 0.3M NaCl and 10% glycerol, and
passing it through a 0.7x20 cm DEAE-Sephadex column equilibrated with hypotonic buffer containing 0.3M NaCl and 10% glycerol (this S100 supernate treated with DEAE-Sephadex is called DE-S100). Normally three or four mice were sacrificed for each enzyme preparation yielding about 50ml of DE-S100. Part of this material was dialyzed overnight (with one buffer change) against 250ml of hypotonic buffer containing 50% glycerol. The enzyme was stored at -20° as the ribose trans-methylase activity was stable under these conditions (this material was called GD-S100). The same procedure was used for the preparation of mouse liver extracts. Livers were obtained from ICR mice, perfused with cold saline, forced through a tissue press, and homogenized in 4.5ml of hypotonic buffer per gram of liver. Preparations of mouse liver S100, DE-S100, and GD-S100 were as described for Ehrlich ascites cells.

3. Methyl-Deficient RNA from Ethionine Treated Mice

ICR mice (8 weeks old, about 40g each) were injected intraperitonally with L-ethionine (125 mg/kg body weight) and adenine (120mg/kg body weight). The injections (about 1ml total volume)
were repeated after 24 hours, and the animals were sacrificed after another 24 hours (16). The livers were perfused with cold saline and stored in cold saline for no more than an hour. The livers were forced through a tissue press, homogenized in hypotonic buffer (4.5 ml/g tissue) and the S\textsubscript{100} fraction was obtained as previously described. The S\textsubscript{100} fraction was used as a source of methyl-deficient tRNA and the 100,000\text{g} pellet was used as a source of methyl-deficient rRNA. The RNA was extracted as described below.

4. Isolation of RNA

RNA was isolated from the livers of ethionine treated mice, untreated mice, and from Ehrlich ascites cells and then used as substrate for Ehrlich ascites and mouse liver transmethylases. S\textsubscript{100} supernates were obtained as previously described and then mixed with 67 volumes of sodium dodecyl sulfate buffer (SDS) buffer (0.14 M NaCl, 0.05 M Na acetate, 0.3\% w/v SDS, pH 5.1) and then with 67 volumes of phenol-cresol solution (75\% phenol, 16\% cresol, and 9\% H\textsubscript{2}O v/v, 1g/liter 8-hydroxyquinoline). This mixture was heated to 55\° with continuous stirring, and then chilled to room temperature in an ice-bath. Stir-
ring was continued for an additional 30 minutes at room temperature and the phases were separated by centrifugation at 3,000xg(6). The aqueous phase was collected, RNA was precipitated with two volumes of 95% ethanol (2% potassium acetate) at -20° for 8 hours, pelleted by centrifugation (3,000xg for one hour) and finally washed with 95% ethanol. The RNA pellet was dissolved in autoclaved water and the concentration was determined by absorbance at 260 nm (E1%=200).

5. The Transmethylase Assays

The general strategy in the transmethylase assay was to incubate, transmethylase extract, RNA, and [(3H)-methyl]SAM; then, to recover the RNA and analyze its 3H-methylated components. The assay mixture contained 100ul enzyme extract (in hypotonic buffer with 0.1M KCL), 1.4uM [(3H)-methyl]SAM [specific activity 12-14 Ci/mmole], and 200ug RNA (total reaction volume was 150ul) were mixed in a 1.5ml microcentrifuge tube and incubated at 37° for various times. After this incubation, 500ul SDS buffer and 500ul phenol-cresol mixture were added to the reaction, mixed for 30 minutes, and then centrifuged to separate the organic and aqueous phases. Four
hundred microliters of the aqueous phase were removed, mixed with two volumes of 95% ethanol (2% potassium acetate), and kept at -80° for 15 minutes. The RNA was pelleted at 15,500xg in an Eppendorf microcentrifuge, dissolved in 100ul water, and precipitated with three volumes of 95% ethanol (2% potassium acetate). The RNA was isolated by centrifugation, washed in 95% ethanol, and allowed to dry. This RNA was free of unincorporated radioactivity and ready for the analysis of the (3H)-methylated components (figure 1).

Total transmethylase activity was determined by dissolving suspending the precipitated RNA in 500ul of water and 5.5ml of Scintiverse E. Then radioactivity was determined by scintillation counting. Ribose transmethylase was determined by two techniques described below and summarized in figure 1.

5a. The Filter Binding Assay

The RNA isolated from the incubations was digested with 1 unit (10ul) of T2 ribonuclease (50mM ammonium acetate, pH 4.5 buffer), for four hours at 37° (22), followed by 0.15 units (40ul) of alkaline phosphatase (0.1M Tris-HCl, pH 8.3) for another four hours. These diges-
determine total radioactivity = total transmethylase

\[ ^3H-RNA \text{ T}^2 \text{ RNase} \ Nmp^{(-2)} + NmpNp^{(-3)} \]

DEAE-Sephadex Column

\[ Np^{(-2)} + NmpNp^{(-3)} \rightarrow Np^{(-2)} + Nmp^{(-3)} \]

Filter Binding Assays

alkaline phosphatase

\[ N^{(0)} \rightarrow Nmp^{(-1)} \]

Apply to DEAE filter and rinse

\[ N^{(0)} \rightarrow NmpN-1 \] (free) (bound)

Elution by a 0 - 0.3M NaCl gradient

\[ Np^{(-2)} + NmpN^{(-3)} \]

cpm

fraction

count filters

**figure 1**
Assays of In Vitro Transmethylase Activity:
Total Transmethylase, DEAE-Sephadex Chromatography and Filter Binding Assays
tion products (nucleosides, NmpN's and larger oligomers) were diluted to 5ml with deionized water and applied to Whatman DE-81 filters (2.4cm diameter) at a flow rate of approximately one milliliter per minute using a Hoefner filtration manifold. The filtrates were collected in test tubes and recycled through the filters four times. The filters were washed twice with 95% ethanol (to remove residual salt) and twice with 0.5mM NaCl. A preliminary experiment had determined that under these conditions NmpN,s or larger oligomers were retained on the filters but nucleosides were removed. In addition, a (\( ^3 \)H)-RNA control sample with 3% endogenous ribose methylation was assayed in each experiment to confirm that the RNA was completely digested and that nucleosides were completely washed from the filters (see results, section 13).

5b. DEAE-Sephadex Chromatography

Characterization of Digestion Products

DEAE-Sephadex chromatography is a commonly used technique for separating various size oligonucleotides(23). The phosphodiester linkages of ribose-methylated nucleotides are resistant to alkaline hydrolysis, i.e. they can be identified
as dinucleotides or larger oligonucleotides in RNA hydrolysates. At pH 7.5, mononucleotides have a charge of -2 and are eluted from the column by a salt gradient before the dinucleotides which have a charge of -3.

The (³H)-RNA, isolated from incubation mixtures, was dissolved in 500ul of 0.3N NaOH and digested at 37° for 16 hours. The sample was diluted with 45ml of urea buffer(7M urea, 0.025M Tris-HCl, pH 7.5), the pH was adjusted to 7.5, and then applied to a 0.7x20 cm DEAE-Sephadex column. A 200ml 0-0.3M NaCl gradient made in urea buffer(23) was used to elute the digestion products. Fractions were measured for absorbance at 260 nm, 1.5ml of each fraction was mixed with 5.5ml of Scientiverse E scintillation cocktail, and radioactivity was determined by scintillation counting.

6. Isolation of Nuclei from Ehrlich Ascites Cells

Nuclei were isolated from Ehrlich ascites cells according to Higashi et.al.(24). Ehrlich ascites tumor cells (see section 1) were washed in hypotonic buffer to lyse red blood cells, pelleted at 250xg, suspended in 10 volumes of a
modified hypotonic buffer (pH 7.6 instead of pH 8 and containing 0.2 % Nonidet NP-40), and homogenized in a glass homogenizer using four strokes of a motor-driven teflon pestle at 1,000rpm. The crude nuclei were pelleted at 500xg and suspended in ten volumes of sucrose buffer (.34M sucrose, 10mM MgCl₂, 10mM Tris-HCl, 10mM NaCl, 1mM DTT, pH 7.6), then 35ml-aliquots of this suspension were layered over 15ml of 0.88M sucrose buffer (same as above except 2.0mM MgCl₂ and 0.88M sucrose) in 50ml conical centrifuge tubes and centrifuged at 2,000xg for ten minutes to pellet the nuclei. The supernates and sucrose cushions were carefully removed to avoid contaminating the nuclei and the pellets were suspended in hypotonic buffer (2.66ml/gram cells) using a glass homogenizer and five strokes of a motor-driven teflon pestle at 1,000rpm. The nuclei were considered to be sufficiently free of cytoplasmic contamination if no "cytoplasmic tags" were visible when the nuclear sample was viewed with a phase contrast microscope at 100x.

The purified nuclei were disrupted at 0° by alternating ten second sonications from a Branson sonicator (maximum power) with one minute pauses to allow for cooling (the procedure was repeated
eight times). The nuclear suspension was then centrifuged at 100,000xg for two hours and the supernate (high speed nuclear extract) was used as a source of ribose transmethylase.

7. Purification of Ribose Transmethylase from Ehrlich Ascites Cells

ICR mice bearing Ehrlich ascites tumor cells were used to prepare a nuclear extract as described in section 5. This extract had a high ratio of ribose to base transmethylase activity when compared to the whole cell homogenate; therefore, the isolation of nuclei represents a significant purification of ribose from base transmethylase. The ribose transmethylase was purified using DEAE-Sephadex chromatography (which also removes endogenous nucleic acid), ammonium sulfate precipitation, and Sephacryl S-300 chromatography. Matrex Gel Green A chromatography was used in one experiment to remove the remaining base transmethylase (10% of the total transmethylase) from a ribose transmethylase preparation purified through the Sephacryl S-300 step.
8. Analysis of Alkaline Resistant Material

8a. Perchloric Acid Digestions

When \((^{14}\text{C})\)-methyl methylated RNA is digested with 70% perchloric acid, the \(2'-0-(^{14}\text{C})\)-methyl groups are converted to \((^{14}\text{C})\)-methanol\((24)\), a volatile compound, but none on the \((^{14}\text{C})\)-methyl groups on the bases are converted to volatile products. The radioactivity removed by lyophilization, after digesting the sample with perchloric acid originates from \((^{14}\text{C})\)-methyl ribose and the remaining radioactive material is from the base methylated \((^{14}\text{C})\)-methyl nucleotides. Twenty milligrams of methyl-deficient tRNA was incubated with 1\muM \((^{14}\text{C})\)-SAM\((50\text{mCi/mmole})\) in 10ml of purified ribose transmethylase for one hour. The \((^{14}\text{C})\)-RNA was isolated as previously described and hydrolyzed in 4ml of 0.3N NaOH at 37\(^\circ\) for 16 hours. The digestion products were separated by DEAE-Sephadex chromatography as described in section 4b. The fractions containing radioactive ribose methylated oligomers were combined and diluted ten-fold with water and applied to a 0.7x4.0 cm column containing 1.0ml of DEAE-Sephadex. The column was washed with 500ml of water followed by 50ml of 0.02M triethylamine
carbonate and finally the bound radioactive material was eluted with 2.0ml of 2.0M triethylamine carbonate. The samples were diluted two-fold with water and evaporated to dryness under a vacuum, suspended in a milliliter of water, and evaporated to dryness two more times. This sample, containing ribose methylated oligomers, had 1,390cpm of radioactivity.

Twenty milligrams of *E. coli* tRNA was incubated with 10ml of Ehrlich ascites DE-S100 and 4μM (14C)-SAM (50mCi/mmole) for one hour and the (14C)-RNA was processed as described above except that the mononucleotide fraction (the only radioactive fraction detected) was collected. This sample, containing base methylated nucleotides, had approximately 50,000cpm of radioactivity. *E. coli* tRNA was used in these experiments because it was a good substrate for base transmethylyases.

The dried RNA samples from the reactions described above were suspended in 200μl of 70% perchloric acid, sealed in capillaries, and heated at 100° for an hour(25). The glass capillaries were placed in a dry ice-ethanol slurry, opened, and the digested samples were transferred to 1.5ml microcentrifuge tubes. The volatile
digestion products, and the perchloric acid were removed under vacuum. One milliliter of methanol was added to the samples and evaporated (this was repeated three times). $H_2^{32}PO_4$ was added to the samples prior to digestion in order to estimate the fraction of radioactivity lost due to physical manipulations. $(^{14}C)$-SAM or $H_2^{32}PO_4$ was added to control samples (digested and undigested) and aliquots were counted. The perchloric acid digestion did not affect scintillation counting of either isotope.

8b. Enzymatic Digestions

Methyl-deficient mouse tRNA was incubated with Ehrlich ascites DE-S100 and $(^3H)$-methyl SAM then the $(^3H)$-RNA was extracted and divided into three equal samples (30,000cpm, 200ug RNA); each of the these samples was digested with different enzymes to determine if $(^3H)$-DNA was present as a contaminant. A control sample was digested for 4 hrs with one unit of T2 RNase (10ul of 50mM ammonium acetate, pH 4.5 buffer)(22). The second sample was digested with T2 RNase followed by digestion with 15 units of DNase I (1.0ml of 10mM Tris-HCl, 3.3mM MgCl$_2$, pH 7.6 buffer) for 6 hours(26). A third sample was digested with T2 RNase, with 0.15 units of alkaline phospha-
tase (40ul of 0.1M Tris-HCl, pH 8.3) for 4 hours, and with 0.8ug of snake venom phosphodiesterase (40ul of 0.1M Tris-HCl, 0.01M MgCl$_2$, pH 9.0) for four hours (22). All digestions were at 37°. The digestion products were applied to separate DEAE-Sephadex columns (section 4b) for analysis.

9. DEAE-Sephadex Column Chromatography for Determining the Size of Alkaline Resistant RNA Fragments

The size of the ribose-methylated oligonucleotides is an indication of the number of adjacent bases that are methylated by the ribose transmethylase. Ten milligrams of yeast RNA was digested with T1 RNase (150 units enzyme in 2ml of 0.02M sodium phosphate buffer, pH 7.5, 37°) for 24 hours. These T1 RNase digestion products were combined with (3H)-methyl RNA previously hydrolyzed with 0.3N NaOH and applied to a 1x75 cm DEAE-Sephadex column equilibrated with urea buffer. The column was washed with 500ml of urea buffer and the RNA fragments bound to the column were eluted with a 2 liter 0.05-0.4M NaCl gradient in urea buffer and 5.0ml fractions were collected (27). The absorbance at 260nm and the radioactivity of each fraction were determined.
10. **Protein Determination**

Protein was determined by the Lowry method (28) and the Coomassie dye binding method (29) using bovine serum albumin as a standard.

11. **SDS-PAGE**

Protein samples were analyzed by SDS-PAGE (30). Twenty micrograms of protein (except for the sample obtained from the Matrex Green Gel A column; see results) were applied to 15x17 cm slab gels (BRL vertical gel apparatus) and electrophoresed at 100 mAmps. The following protein standards (obtained from Biorad) were electrophoresed in a separate lane: Lysozyme (14,000 daltons), Soybean Trypsin Inhibitor (21,500 daltons), Carbonic Anhydrase (31,000 daltons), Ovalbumin (45,000 daltons), Bovine Serum Albumin (66,200 daltons), Phosphorylase B (92,500 daltons), B-Galactosidase (116,250 daltons) and Myosin (200,000 daltons). Protein bands were stained using silver stain (31).
RESULTS

11. Assay of Transmethylase Activity

I demonstrated in vitro ribose and base transmethylase activities by incubating Ehrlich ascites cell supernate with \(^{3}H\)-SAM and various RNA substrates. Total transmethylase was determined by scintillation counting the recovered \(^{3}H\)-RNA. Two techniques were used to quantify radioactivity incorporated into ribose and bases (figure 1, section 5). The first technique, DEAE-Sephadex chromatography, was used only for non-routine analysis and a second technique, a filter binding assay, was used as a routine enzyme assay. In the DEAE-Sephadex chromatography technique, RNA hydrolysates were separated into base-methylated mononucleotides (Np) and ribose-methylated dinucleotides (NmpNp) and larger oligomer fractions. The general strategy in the ribose transmethylase filter binding assay was to degrade \(^{3}H\)-RNA to nucleosides and NmpNs, to bind these products to DEAE filters, and then to wash off the nucleosides and determine the radioactivity in the NmpNs bound to the filters.

Under conditions outlined in the methods, an initial control experiment established that NmpNs
were retained on the filters but nucleosides were removed. In this experiment nucleotides and dinucleotides were isolated by DEAE-Sephadex chromatography and digested with alkaline phosphatase to produce samples of nucleosides and NmpN. Four equal aliquots of Np were digested with alkaline phosphatase to produce nucleosides which were applied to DEAE-filters; two filters were washed, two were not. The unwashed filters had 2100cpm and the washed filters had 18cpm (background subtracted). The same procedure was repeated for NmpNp digested with alkaline phosphatase and all four filters, washed or unwashed had 390cpm. The (³H)-RNA used in this preliminary experiment was previously determined, by DEAE-Sephadex chromatography, to have 3% of the total radioactivity in the dinucleotide fraction. The T₂-alkaline phosphatase digestion products should be 97% nucleoside and 3% NmpN; therefore, 97% of the total radioactivity should be removed from DEAE filters. This (³H)-RNA was analyzed by the filter binding assay many times, and 97% of the radioactivity applied to DEAE filters was consistently removed by washing. Typical results were 43,431cpm on unwashed filters and 1,301cpm on washed filters. As a control in all subsequent
experiments, this \(^{3}H\)-RNA was mixed with 200\(\mu\)g of nonradioactive RNA and analyzed by the filter binding assay along with the other samples. If more than 3% of the total radioactivity in this control sample bound to the filter, it was assumed that either the RNA was not completely digested or nucleosides were not completely washed from the filters. Thus these data showed that the filter binding assay measured radioactivity in \(^{3}H\)-methyl ribose.

12. Characterization of DE-S100 and GD-S100 Fractions

Endogenous RNA was removed from crude transmethylase by adjusting the S100 to 0.3M NaCl and passing it over a DEAE-Sephadex column, followed by dialysis to remove the NaCl. DE-S100 was extracted with phenol-creosol mixture (section 3, RNA isolation) but no RNA was precipitated from the aqueous phase by the addition of 95% ethanol (2% potassium acetate) because there was no visible RNA pellet or soluble material which absorbed light at 260 nm. DE-S100 incubated with \(^{3}H\)-SAM but without exogenous RNA had no incorporated radioactivity but an identical sample incubated with 200\(\mu\)g of exogenous RNA had
37,820 cpm (total transmethylase). Since small amounts of degraded endogenous RNA might not precipitate under these conditions, 200 μg of carrier RNA was added to the sample after the incubation was terminated. This sample had 3% (1160 cpm) of the radioactivity of the complete incubation. A heat-inactivated enzyme control (DE-S100 boiled before the assay) showed no activity.

DE-S100 and GD-S100 were assayed for ribose and base transmethylase activities by the filter binding assay using the standard assay mixture (100 μl enzyme, 1.4 μM (3H)-SAM and 200 μg Ehrlich ascites tRNA) and the mixture was incubated for 45 min at 37°C. Previous experiments, analyzing 1.0 ml samples by the DEAE-Sephadex column technique (see Methods), demonstrated that the ribose transmethylase activity in DE-S100 was consistently 13% of the total RNA transmethylase activity. Ribose and base transmethylase activities in DE-S100 and GD-S100, determined by filter binding assays, confirmed that ribose transmethylase was about 13% of the total RNA transmethylase activity in DE-S100 (typical values were 1317 cpm for ribose methylation and 7929 cpm for base methylation.)
13. Comparison of Transmethylase Activities in Ehrlich Ascites Cells and Mouse Liver

Ehrlich ascites whole cell homogenates, S100 extracts and DE-S100 extracts had a two to three-fold elevated RNA transmethylase activity compared to the corresponding fractions from mouse liver when methyl-deficient mouse tRNA was used as a substrate. The GD-S100 extracts from both tissues were used for additional comparative experiments described below.

The standard assay was used with Ehrlich ascites or mouse liver GD-S100 containing 0.75mg of protein. Samples from each tissue were incubated for the various times and then processed as described in section 2 to determine total methylation and ribose methylation. Ehrlich ascites GD-S100 had a ten-fold elevated total transmethylase activity compared to mouse liver when E. coli tRNA was used as a substrate (figure 2), but only a two-fold elevated total transmethylase activity when methyl-deficient mouse liver tRNA was used as a substrate, (figure 3). DEAE-Sephadex column chromatography and filter binding assays indicated that only base methylation was occurring on E. coli tRNA (figure 4) and that both base and ribose methylation was occurring on
The incubation contained 0.23 nmoles of $^3$H-methyl SAM, 200 ug of E. coli tRNA, 0.75 mg of protein [from Ehrlich ascites (-□-) or mouse liver (-X-) GD-S100] and 120 ul of hypotonic buffer in a total volume of 160 ul. The RNA was extracted and the radioactivity in each sample was determined by scintillation counting.
Figure 3
Total Transmethylase Activity of Ehrlich Ascites and Mouse liver Extracts on Methyl-Deficient Mouse tRNA

The incubation contained 0.23 nmoles of $^3$H-methyl SAM, 200 ug of methyl-deficient mouse tRNA, 0.75 mg of protein [from Ehrlich ascites(-□-) or mouse liver(-X-) GD-S100] and 120 ul of hypotonic buffer in a total volume of 160 ul. The RNA was extracted and the radioactivity was determined by scintillation counting.
E. coli $^3$H-tRNA, methylated by incubation with cell extract and $^3$H-SAM, were isolated from incubation mixtures, was dissolved in 0.3N NaOH and digested at 37° for 16 hours. The sample was diluted with enough urea buffer (7M urea, 0.025 M Tris, pH 7.5) to bring the pH to 7.5 and then applied to the DEAE-Sephadex column. A 200 ml 0 -0.3M NaCl gradient made in urea buffer was applied to the column and collected in fractions which were measured for absorbance at 260 nm (not shown) and for radioactivity. Mononucleotides eluted in fractions 28-40.
methyl-deficient mouse tRNA (figure 5). The base and ribose methylation activities were elevated two-fold in Ehrlich ascites cells (figures 3 and 6) when compared to mouse liver. However, when mouse liver or Ehrlich ascites tRNA (methylated at normal levels) was used as substrate they only had 10% the methyl-accepting capacity of methyl-deficient mouse tRNA when incubated with either Ehrlich ascites or mouse liver GD-S100.


When (3H)-methyl RNA was isolated from a methyltransferase incubation, and hydrolyzed and the products were separated by DEAE-Sephadex column chromatography, some radioactive material was eluted by a higher salt concentration than the dinucleotides. These alkaline resistant oligonucleotides seem to be produced by successive ribose methylations; however, this material could also be small DNA fragments contaminating the methyl-deficient mouse tRNA and acting as a transmethylase substrate.

The experiments designed to identify this alkaline resistant material are described below.
Methyl-deficient mouse $^3$H-tRNA, isolated from incubation mixtures, was dissolved in 0.3 N NaOH and digested at 37° C for 16 hours, and then applied to a DEAE-Sephadex column as described in figure 4. Mononucleotides eluted in fractions 28-38, dinucleotides in fractions 44-50 and oligonucleotides in fractions above 54.
Determination of \textit{In Vitro} Ribose Methylation of Methyl-Deficient Mouse $^3$H-tRNA by Filter Binding Assays

Methyl-deficient mouse tRNA was incubated with Ehrlich ascites (-□-) or mouse liver (-X-) GD-S100 and then the $^3$H-tRNA was isolated and digested with 10 ul of T2 ribonuclease for four hours at 37°C. Then 40 ul of alkaline phosphatase was added and the incubation was continued for another four hours. These digestion products, nucleosides and NmpNs, were diluted to 5 ml with deionized water and filtered through Whatman DE-81 circle filters using a Hoeffer filtration manifold and the filtrates were collected in test tubes. The filtrates were recycled through the filters four times and then the filters were allowed to dry. The dry filters were washed twice with ethanol, then twice with 0.5 mM NaCl.
14a. Analysis of Unhydrolyzed (³H)-methyl tRNA
   by DEAE-Sephadex Chromatography

Methyl-deficient mouse tRNA was incubated with Ehrlich ascites GD-S100 and (³H)-SAM and then extracted from the mixture. The intact (³H)-methyl RNA, i.e. not hydrolyzed, was applied to a DEAE-Sephadex column. When a 0.0-0.3M NaCl gradient was used to elute small fragments of nucleic acid, no radioactive material was eluted from the column. This experiment demonstrated that there were no small DNA fragments either in the RNA sample or the GD-S100 acting as a trans-methylase substrate.

14b. Enzymatic Digestions on (³H)-methyl tRNA

Equal amounts of (³H)-methyl RNA were digested with T2 RNase, or T2 RNase plus DNase I, or T2 RNase plus snake venom phosphodiesterase as described in the methods, to determine if DNA was present in the T2 RNase products. Then the products of these three enzymatic digestions were applied to separate DEAE-Sephadex columns and eluted with a salt gradient (figure 7) as described in section 3. There was no difference between the elution profiles of the T2 RNase digestion products and the T2 RNase-DNase diges-
Methyl-deficient mouse tRNA was incubated with Ehrlich ascites DE-S100 and $^3$H-methyl SAM then the RNA was extracted and divided into three equal samples. One sample was digested for 4 hrs in one unit of T2 RNase, the second sample was digested with T2 RNase then with 15 units of DNase I for 6 hours and a third sample was digested with T2 RNase then with 0.15 units of alkaline phosphatase for 4 hours and finally with 0.8 ug of snake venom phosphodiesterase for four hours. All digestions were at 37°. The products of these digestions were separated by DEAE-Sephadex chromatography. [Control and DNase(--), phosphodiesterase(—).] Mononucleotides eluted in fractions 30-40; dinucleotides and oligonucleotides eluted in fractions above 46. The continuous lines actually represent individual points.
tion products (figure 7) indicating that the DNase did not degrade any fragments. [90% of the calf DNA in a control T2 RNase-DNase digest was converted to fragments which eluted from the DEAE-Sephadex column at a lower salt concentration than the largest T2 RNase fragments (data not shown)].

Snake venom phosphodiesterase had considerable hydrolytic activity on the T2 RNase plus alkaline phosphatase digestion products because most of the radioactive material in the T2 RNase-phosphodiesterase sample (nucleosides) did not bind to the DEAE-Sephadex column and the bound radioactive material eluted at a lower salt concentration than the T2 RNase fragments (figure 7). The sensitivity of T2 RNase products to snake venom phosphodiesterase (5' exonuclease) indicates that all of the radioactive material was nucleic acid and the insensitivity of the T2 RNase products to DNase indicates the material was not DNA but RNA.

14c. Perchloric Acid Digestion of

\( ^{14}\text{C} \)-methyl Nucleotides and Oligomers

Perchloric acid digestions were used to identify 2'-O-methyl ribose (see section 8a). Three
samples of comparable radioactivity, one containing only \((^{14}\text{C})\)-methyl alkaline resistant material and two containing only \((^{14}\text{C})\)-methyl bases were digested with perchloric acid. Eighty percent of the radioactivity in the alkaline resistant material was volatilized by the perchloric acid digestion and subsequent lyophilization compared to only 10% and 22% for the two base methylated samples. The low level of radioactivity in the ribose methylated sample (1390cpm) was due to the low specific activity of the \((^{14}\text{C})\)-SAM (50mCi/mmmole). The two base methylated samples were adjusted to comparable levels of radioactivity for a direct comparison with the ribose methylated sample. Two base methylated samples with higher levels of radioactivity were also used in the experiment and almost all the radioactivity present in these samples could be recovered after the digestion and lyophilization (table 1).

The high percentage of radioactivity volatilized by perchloric acid digestion supported the theory that the radioactive species was \(^{14}\text{C}\)-methyl ribose. The perchloric acid technique was used here as a qualitative tool. It might have been developed further and used as a quanti-
Table 1
Perchloric Acid Digestions

<table>
<thead>
<tr>
<th></th>
<th>Radioactivity Before Digestion (cpm)</th>
<th>Radioactivity After Digestion (cpm)</th>
<th>Percent of Radioactivity Not Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>^14C-methyl Base Methylated Samples</td>
<td>1,730</td>
<td>1,346</td>
<td>22</td>
</tr>
<tr>
<td>^14C-methyl Ribose Methylated Sample</td>
<td>1,750</td>
<td>1,570</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>10,470</td>
<td>10,482</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>9,940</td>
<td>9,523</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>1,390</td>
<td>285</td>
<td>80</td>
</tr>
</tbody>
</table>

Methyl-deficient tRNA was incubated with ^14C-SAM and purified ribose transmethylase, the RNA was extracted, hydrolyzed with 0.3 N NaOH, and then the products were separated by DEAE-Sephadex chromatography as previously described. Radioactive material from the mononucleotide peak and from the high molecular weight peak was isolated and dried in microcentrifuge tubes. The dried material was suspended in 200 ul of 70% perchloric acid, sealed in glass capillaries, and heated at 100° for an hour. The volatile digestion products, as well as the perchloric acid, were removed under a vacuum by adding one milliter of methanol to the samples which were then evaporated to dryness; this procedure was repeated three times.
tative tool (100% volatilization of methyl ribose) but this was not done because there were two other techniques at hand for quantifying methyl ribose.

15. Determining the Length of Ribose Methylated RNA Fragments

Methyl-deficient mouse tRNA was incubated with Ehrlich ascites GD-S100 and (3H)-SAM and then the (3H)-RNA was extracted and hydrolyzed with 0.3 N NaOH. Radioactivity from alkaline hydrolyzed (3H)-methyl RNA coeluted from a DEAE-Sephadex column with T1 RNase fragments of one, two, four and five bases in length (figure 8). The radioactivity which coelutes with the mononucleotide peak represented nucleotides with methylated bases and the radioactivity coeluting with T1 RNase fragments two or more nucleotides in length represented RNA fragments with methylated ribose. Radioactivity eluting after the dinucleotide peak reflect the tendency of the transmethylase to methylate adjacent residues and may represent a processive activity of the ribose transmethylase. The radioactivity coeluting with the four and the five nucleotide T1 fragments indicated that the enzyme was methylating no more
DEAE-Sephadex Column Chromatography for Determining the Size of Alkaline Resistant RNA Fragments

Yeast RNA (10mg) was digested with T1 RNase (150 units enzyme in 2 ml of 0.02 M sodium phosphate buffer, pH 7.5, 37°) for 24 hours. These T1 RNase digestion products were combined with 3H-methyl RNA previously hydrolyzed with 0.3 N NaOH and applied to a 1 cm x 75 cm DEAE-Sephadex column equilibrated with urea buffer. The column was washed with 500 ml of urea buffer. The RNA fragments were eluted with a 2 liter 0.05 -0.4 M NaCl gradient in urea buffer and collected in 5.0 ml fractions; then the absorbance at 260 nm and the radioactivity of each fraction was determined. [T1 fragments(A260)(---), cpm(-----)]. Numbers represent the chainlength of RNase fragments.
than three or four residues.

16. Purification of Ribose Transmethylase

16a. DEAE-Sephadex Chromatography

The high speed nuclear extract was adjusted to 0.3M NaCl, 2mM EDTA, applied to a 0.7cm x 14cm DEAE-Sephadex column previously equilibrated with hypotonic buffer lacking MgCl₂, and the unab- sorbed material was collected. This procedure removed endogenous nucleic acids from the supernate and some protein.

16b. Ammonium Sulfate Precipitation

The unabsorbed material from the DEAE-Sephadex column was adjusted to 20% saturation of ammonium sulfate by adding solid ammonium sulfate with continuous stirring at 0°. A drop of the mixture was applied to pH sensitive paper which indicated that the pH was between 7.8 and 8.0. The sample was kept on ice for an additional 20 minutes after all the ammonium sulfate had dissolved, and then centrifuged at 10,000xg. The supernatant solution was adjusted to 60% saturation of ammonium sulfate, kept on ice for an hour after the ammonium sulfate had dissolved and cen-
trifuged for 20 minutes at 10,000xg. The supernate was discarded (previous experiments had determined that the transmethylase activity was precipitated at 60% saturation) and the precipitated protein was dissolved in 2.0ml of EDTA buffer (10mM Tris-HCl, 0.1M KCl, 1mM DTT, 0.2mM EDTA, pH 8.0) to a protein concentration of about 3.0 mg/ml and glycerol was added to 5% v/v.

16c. Sephacryl S-300 Gel Filtration

Chromatography

The suspended protein from the ammonium sulfate precipitation(2-3ml) was applied to a 3x40 cm Sephacryl S-300 column previously equilibrated with EDTA buffer at 4° and pumped at a flow rate of 100ml/hour (15ml/hour/cm cross sectional area). Two ml fractions were collected, and every other fraction was assayed for total RNA transmethylase activity. Total RNA transmethylase vs fraction number was plotted (figure 9) and all assayed and unassayed fractions falling under the major activity peak (fractions 42-68) were pooled as were the fractions under the minor peak (fraction 33-40). The pooled fractions were then
The protein from the ammonium sulfate precipitation was dissolved in EDTA buffer and applied to a 3 x 40 cm Sephacryl S-300 column previously equilibrated with EDTA buffer at 4°C and pumped at a flow rate of 100 ml/hour (15ml/hour/cm cross sectional area). 2.0 ml fractions were collected and every other fraction was assayed for RNA transmethylase activity.
assayed to quantify the activity. A portion of each sample was also dialyzed against EDTA buffer in 50% glycerol for storage at -20°, while the rest of the sample was applied to a Matrex Gel Green A column.

16d. Matrex Gel Green A Chromatography

A portion of the pooled fractions from the Sephacryl S-300 column (the major activity peak, 40ml) was adjusted to 20% glycerol, applied to a Matrex Gel Green A column previously equilibrated with EDTA buffer, and washed with 20ml of EDTA buffer adjusted to 0.25M KCl. Then the RNA transmethylase was eluted with EDTA buffer adjusted to 2M KCl. This step did not result in additional purification of the transmethylase because all of the protein bound to the dye column and eluted with the transmethylase. There remained the possibility that Matrex Gel Green A chromatography would be a useful purification step if a salt gradient was used to elute the activity instead of a single salt concentration. A purified transmethylase sample (the Sephacryl-300 fraction) was adjusted to 50% glycerol and stored at -20°. This stored transmethylase sample was diluted with one volume of EDTA buffer and applied to the Matrex
Gel Green A column. The column was washed with 50ml of EDTA buffer, and the transmethlylase activity was eluted with a 100ml 0.1-2.1M KCl linear gradient in EDTA buffer, 20% glycerol. Three milliliter fractions were collected, 6ul were removed from each fraction and diluted to 125ul with EDTA buffer and assayed. The fractions containing the majority of RNA transmethylase activity (fractions 3 to 8) were pooled (figure 10), dialyzed overnight and assayed the next day for RNA transmethylase activity using the DEAE-Sephadex column technique to distinguish base from ribose methylation (figure 11). No base transmethylase activity could be detected in this preparation of ribose transmethylase.

16.e The Purification of tRNA Ribose Transmethylase

The purification procedure described above (through the Sephacryl S-300 step) yielded an 910-fold partially purified tRNA ribose transmethylase (table 2) which contained only 10% base transmethylase activity (figure 12). The low percentage of base transmethylase in this partially purified sample facilitated the characterization of the ribose transmethylase with nominal inter-
The pooled fractions (42-68) from the Sephacryl S-300 column (45 ml) were adjusted to 20% glycerol, applied to a Matrex Gel Green A column previously equilibrated with EDTA buffer and the column was washed with 20 ml of EDTA buffer. The RNA transmethylase activity was eluted with a 100 ml 0.10–2.1 M KCl gradient made in EDTA buffer, 20% glycerol. Three milliliter fractions were collected and the fractions containing RNA transmethylase activity were pooled. The pooled fractions were dialyzed overnight and assayed the next day for ribose and base transmethylase activity.
Figure 11.
DEAE-Sephadex Analysis of Ribose Transmethylase
Eluted from a Matrex Gel Green A Column

Ribose transmethylase eluted from the Matrex Gel Green A Column (figure 10) was assayed for ribose and base transmethylase activity (methyl-deficient mouse tRNA was used as substrate) by the DEAE-Sephadex chromatography technique. Mononucleotides would normally elute in fractions 30-40, dinucleotides in fractions 45-52, and oligonucleotides in fractions above fraction 52.
Table 2

<table>
<thead>
<tr>
<th></th>
<th>Total Protein (mg)</th>
<th>Total Activity (cpm x 10^-6/45 min)</th>
<th>Specific Activity (activity x 10^-6/mg protein)</th>
<th>Fold Purification</th>
<th>% Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>657</td>
<td>4.2</td>
<td>0.64</td>
<td>-----</td>
<td>100</td>
</tr>
<tr>
<td>Nuclear Suspension</td>
<td>97.2</td>
<td>3.5</td>
<td>3.6</td>
<td>5.6</td>
<td>84</td>
</tr>
<tr>
<td>Nuclear Extract</td>
<td>7.21</td>
<td>2.8</td>
<td>39</td>
<td>61</td>
<td>68</td>
</tr>
<tr>
<td>DEAE-Sephadex</td>
<td>not assayed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium Sulfate</td>
<td>2.93</td>
<td>4.2</td>
<td>140</td>
<td>220</td>
<td>100</td>
</tr>
<tr>
<td>Sephacryl 300</td>
<td>0.71</td>
<td>4.1</td>
<td>580</td>
<td>910</td>
<td>97</td>
</tr>
</tbody>
</table>
Figure 12
Analysis of Ribose And Base Transmethylase Activities in Partially Purified Ribose Transmethylase

$^3$H-methyl RNA, isolated from incubation mixtures, was dissolved in 0.3 N NaOH and digested at 37° for 16 hours. The sample was then applied to the DEAE-Sephadex column as described in figure 4.
ference from the base transmethylase activity. The absence of radioactivity in fractions 45-50 (figure 12), which would contain dinucleotides, indicates that the purified enzyme generated sequentially methylated fragments in vitro.

17. The Effect of pH on the Reaction Velocity

The effect of varying pH on the reaction velocity of purified tRNA ribose transmethylase was studied at pH 5.7 to pH 6.7 using 10mM [2(N-morpholino)ethane sulfonic acid](MES) buffer and at pH 7.18 to pH 8.5 using 10mM Tris-HCl (figure 13) in the presence of 0.1M KCl. The enzyme activity was maximum at pH 8.0 and decreased by 20% per unit change in pH from 7.5 to 6.7. The enzyme displayed a larger decrease in activity below pH 6.7 or above pH 8.0.

18. The Effect of KCl Concentration on Reaction Velocity

The effect of varying KCl concentration on the reaction velocity of the purified enzyme was studied over the concentration range of 0.0 to 0.20M KCl (figure 14). The enzyme activity was maximum (100%) at 0.1M KCl, 25% in the absence of
Figure 13
The Effect of pH on the Ribose Transmethylase Activity

The effect of varying pH on the reaction velocity (cpm/45min under standard assay conditions) of purified tRNA ribose transmethylase was studied at pH range 5.7 to 6.7 using 10 mM MES buffer and at 7.18 to 8.5 using 10 mM Tris.
Figure 14
The Effect of KCl Concentration on the Ribose Transmethylase Activity

The effect of varying KCl concentration on the reaction velocity (cpm/45 min under standard assay conditions) of the purified enzyme was studied in the range of 0.0 to 0.20 M KCl.
KCl and 57% at 0.2M KCl.

19. **Determination of the Apparent Molecular Weight of tRNA Ribose Transmethylase**

Sephacryl S-300 gel filtration chromatography was described above. The elution profile of tRNA ribose transmethylase activity was compared to the elution profiles (detected by absorbance at 280nm) of four protein molecular weight markers: apoferritin (443,000 daltons), β-Amylase (200,000 daltons), alcohol dehydrogenase (150,000 daltons), and bovine serum albumin (66,000 daltons). All conditions were as in the previously described protocol. Kav of the protein molecular weight markers was plotted versus log molecular weight and a line was determined by visual inspection (figure 15). The Kav for ribose transmethylase was 1.43 which corresponds to an apparent molecular weight of 240,000 daltons.

An SDS-polyacrylamide gel of the ribose transmethylase eluted from the Matrex Gel Green A column showed a predominant band at approximately 60,000 daltons as judged by its migration relative to molecular weight standards (figure 16). This datum suggest that the native ribose transmethylase may exist as a tetramer with four
Figure 15
Determination of the Apparent Molecular Weight of Ribose Transmethylase by Sephacryl S-300 Chromatography

The elution profile of tRNA ribose transmethylase activity was compared to the elution profiles (detected by absorbance at 280nm) of four protein molecular weight markers: apoferritin (443,000 daltons), β-Amylase (200,000 daltons), alcohol dehydrogenase (150,000 daltons), and bovine serum albumin (66,000 daltons). All conditions were as in the previously described protocol (section 15c). The transmethylase is indicated by a solid square. The line was drawn by visual inspection.
Protein fractions were analyzed by SDS-PAGE as described in section 11. The following molecular weight standards were used (far right lane): Lysozyme(14,000 daltons), Soy Bean Trypsin Inhibitor(21,500 daltons), Carbonic Anhydrase(31,000 daltons), Oavalbumin(45,000 daltons), Bovine Serum Albumin(66,200), Phosphorylase B(92,500 daltons), B-Galactosidase(116,250 daltons) and Myosin(200,000 daltons). Protein bands were stained using silver stain(31). The protein fractions are (from right to left): nuclear suspension, pooled fractions from Seph- acryl S-300, high speed nuclear extract, and pooled fractions from Matrex Green Gel A.
60,000 dalton subunits. The mass of protein applied to the gel (the majority of the sample) was not sufficient to produce definitive results and there are likely other proteins in the sample that were not detected on this gel.

20. Determination of an Apparent $K_m(tRNA)$

Nonradioactive SAM was added to the stock ($^3$H)-SAM to adjust the specific activity to 0.5 Ci/ mmole. This ($^3$H)-SAM was mixed with purified ribose transmethylase (final concentration = 20 uM SAM) and varying amounts of tRNA. The sample was incubated at 37°C for 40 minutes (incorporation of radioactivity by the purified enzyme was linear for 40 minutes, data not shown). The RNA was then extracted and the radioactivity incorporated into RNA was determined as previously described. The inverse of the RNA substrate concentration(mg/ml)$^{-1}$ was plotted versus the inverse of the velocity(picomoles/min/mg protein)$^{-1}$ (figure 17). The tRNA ribose transmethylase had an apparent $K_m$ of 20uM tRNA, based on an average tRNA molecular weight of 30,000 daltons, and a maximum velocity of 150 picomoles/min/mg protein. It was not possible to reach saturating concentrations of SAM by further decreasing the
Nonradioactive SAM was added to the stock $^3$H-SAM to adjust the specific activity to 0.5 Ci/ mmole, then the $^3$H-SAM was mixed with purified ribose transmethylase (final concentration = 20 uM SAM) and varying amounts of tRNA and incubated at 37° for 40 minutes. The RNA was then extracted and the radioactivity incorporated into RNA was determined as previously described. The inverse of the substrate concentration (mg/ml)$^{-1}$ was plotted versus the inverse of the velocity (picomoles/min/mg protein)$^{-1}$. The line was determined by a visual inspection.
specific activity of the (3H)-SAM because the assay was at the limits of sensitivity.

21. Determination of an Apparent $K_m_{\text{SAM}}$

Purified ribose transmethylase was incubated with 40uM methyl-deficient tRNA (assuming a molecular weight of 30,000 daltons) and varying amounts of (3H)-SAM (0.5 Ci/m mole) in the concentration range of 0.63 to 5.0uM. The inverse of the SAM concentration (uM$^{-1}$) was plotted versus the inverse of the velocity (picomoles/min/mg protein)$^{-1}$. Ribose transmethylase has an apparent $K_m$ of 12.8uM SAM and a $V_{\text{max}}$ of 210 picomoles/min/mg protein (figure 18). There was insufficient methyl-deficient tRNA available to reach saturating concentrations of RNA. Also, there was data to suggest that residual SDS in the RNA preparation (from the RNA extraction buffer) would interfere with the transmethylase assay at high RNA concentrations. Therefore, the conditions described above were chosen after consideration of these factors.

22. Nucleic Acid Substrate Studies

Methyl-deficient mouse tRNA and E. coli tRNA were compared as substrates for tRNA ribose
Figure 18.
Determination of an Apparent $K_{\text{m,SAM}}$ for the Ribose Transmethylase

Purified ribose transmethylase was incubated with 40 uM methyl-deficient tRNA (assuming a molecular weight of 30,000 daltons) and varying amounts of $^3$H-SAM (0.5 Ci/mmmole) in the concentration range of 0.63 to 5.0 uM. The inverse of the SAM concentration (uM)$^{-1}$ was plotted versus the inverse of the velocity (picomoles/min/mg protein)$^{-1}$. The line was determined by a visual inspection.
Methyl-deficient mouse tRNA, \textit{E. coli} tRNA and methyl-deficient nuclear RNA were compared as substrates for tRNA ribose transmethylase. Ribose transmethylase activity was determined by the DEAE-Sephadex chromatography technique.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total Transmethylase Activity</th>
<th>Ratio of Ribose to Base Methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>methyl deficient mouse tRNA</td>
<td>25,000 cpm</td>
<td>9.0</td>
</tr>
<tr>
<td>\textit{E. coli} tRNA</td>
<td>24,770 cpm</td>
<td>0.1</td>
</tr>
<tr>
<td>nuclear methyl deficient RNA</td>
<td>6,500 cpm</td>
<td>9.0</td>
</tr>
</tbody>
</table>
transmethylase using 200μg of each RNA substrate and conditions described previously. The results are shown in table 3. Maximum ribose methylation occurred on methyl-deficient mouse tRNA and, in comparison E. coli tRNA, had 10% of that maximum value. The ribose transmethylase, purified 910-fold, had a low activity toward E. coli tRNA. The ratio of ribose to base transmethylase activity in this preparation was 90-fold less when E. coli tRNA was used as a substrate than when methyl-deficient mouse tRNA was used, even though 97% of the base transmethylase present in the homogenate had been removed from the purified preparation. Notably, the ribose methylation that did occur appeared to result predominantly from sequential methylations (figure 19).

One cannot distinguish whether this low level of ribose transmethylase activity toward E. coli tRNA was present in the homogenate (but below the limits of detection) or absent in the homogenate (the E. coli methylating activity was strictly an in vitro phenomenon which develops during purification). Methyl-deficient mouse nuclear RNA had 25% of the methyl accepting capacity of methyl-deficient mouse tRNA with the majority of the methyl groups being incorporated into adjacent
DEAE-Sephadex Analysis of Ribose And Base Transmethylase Activities in Partially Purified Ribose Transmethylase Using E. coli tRNA as Substrate

E. coli $^3$H-methyl tRNA, isolated from incubation mixtures, was dissolved in dissolved in 0.3N NaOH and incubated at 37°C for 16 hours then applied to the DEAE-Sephadex column as described in figure 4.
nucleotides. Mouse tRNA, isolated from the livers of mice not treated with ethionine, had 5% of the methyl accepting capacity of methyl-deficient mouse tRNA.

23. Conditions Favoring the 2'-O-ribose Methylation at Adjacent Positions.

Controlled studies of conditions which favor 2'-O-ribose methylation of adjacent positions by the ribose transmethylase were not conducted but data from one experiments suggested that the age of a enzyme preparation is important. A single preparation of high speed nuclear extract was assayed fresh and again after being stored at 0° for 48 hours. The major products of the fresh preparation were 2'-O-methyl-dinucleotides, however, a stored preparation produced predominantly methylated 2'-O-methyl-oligonucleotides. The total ribose transmethylase activity had not changed significantly (figure 20).
A preparation of high speed nuclear extract was assayed fresh and again after being stored at 0° for 48 hours and then the products of both assays were analyzed by the DEAE-Sephadex column technique. [fresh(—), stored(—)].
DISCUSSION

This project might be subdivided into four separate areas namely, (A) the demonstration of an in vitro ribose transmethylase activity using exogenous tRNA as substrate and an "RNA free" Ehrlich ascites extract as an enzyme source, (B) the development of an in vitro assay capable of distinguishing between base and ribose methylation and suitable for assaying multiple samples, (C) comparative studies using this assay to quantify base and ribose transmethylase activities in extracts of Ehrlich ascites cell and mouse liver using a variety of RNA substrates and finally, (D) the development of a procedure for separating the ribose transmethylase from the base transmethylase activities so that the former could be studied with nominal interference from the later.

Gallup(19) had previously demonstrated an in vitro ribose transmethylase activity occurring on endogenous tRNA in Ehrlich ascites cell extracts. The inherent limitations of such a system are obvious: comparison of transmethylase activities in crude extracts might be affected by differences in the methyl-accepting capacities of the
respective endogenous tRNAs and the requirement of retaining endogenous tRNA in the preparation would preclude attempts to purify a transmethylase activity. The removal of endogenous tRNA from Ehrlich ascites cell extracts by DEAE-Sephadex chromatography and subsequently the detection of ribose methylation occurring on exogenous tRNA were prerequisite steps for the study of ribose transmethylase activities in crude extracts and the separation of ribose and base transmethylase activities.

Preliminary experiments demonstrated in vitro ribose methylation occurring in "RNA free" Ehrlich ascites extracts using exogenous Ehrlich ascites tRNA as substrate, but the level of activity (0.016 picomoles/min/mg protein) would be difficult to detect at protein concentrations of less than 1.0mg/ml. This problem was compounded by a relatively high background of base transmethylase activity (0.14 picomoles/min/mg protein). A solution to this problem might be the use of methyl-deficient homologous tRNA as substrate. Transmethylases would have a higher activity in vitro toward substrates that had not already been methylated by homologous transmethylases in vivo. Methyl-deficient tRNA has been
obtained from animals treated with the transmethylase inhibitor, ethionine(16) and this methyl-deficient tRNA has been used as an in vitro substrate for tRNA transmethylases. *E. coli* tRNA has been used as a substrate in some studies involving eukaryotic tRNA transmethylases(5). Some eukaryotic tRNA transmethylases can methylate prokaryotic tRNAs in vitro and since all tRNAs have a similar three dimensional shape it has been proposed that these are a subclass of transmethylases which recognize particular tertiary structures common to most tRNAs and can methylate a specific residue relative to that tertiary structure(32). Prokaryotic tRNA is generally less methylated than eukaryotic tRNA and therefore these corresponding sites are often unmethylated on prokaryotic tRNAs.

In comparing the transmethylase activities in Ehrlich ascites and mouse liver extracts, methyl-deficient tRNA from ethionine treated mice, tRNA from the livers of untreated mice, tRNA from Ehrlich ascites cells and *E. coli* tRNA were used as substrates. Ehrlich ascites extracts had two-fold elevated ribose and base transmethylase activities compared to mouse liver when any of the three mouse tRNAs were used as substrates but a
ten-fold elevated activity when E. coli tRNA was used as a substrate; no ribose methylation of E. coli tRNA was detected (table 3). One basic question resolved by these experiments was whether the apparent elevated transmethylase activity in Ehrlich ascites extracts compared to mouse liver extracts observed by Gallup(19) was due to differences in the enzyme activities or differences in the methyl accepting capacities of the endogenous substrates. When endogenous RNA was removed from the extracts, the Ehrlich ascites had an elevated transmethylase activity regardless of which tRNA was used as a substrate.

In considering these experiments, assaying transmethylase activities in Ehrlich ascites extracts, and similar experiments conducted by Gallup(19) another observation might be made concerning the apparent specificity of ribose and base transmethylases in Ehrlich ascites cells. In vitro ribose methylation was detected on endogenous or exogenous normally methylated tRNA, but the best methyl acceptor was exogenous methyl-deficient tRNA (in vitro ribose methylation is not detected on mouse rRNA or E. coli tRNA). Base methylation was occurring on all normally methylated mouse RNA substrates, and on methyl-
deficient mouse tRNA, but the best methyl acceptor was *E. coli* tRNA. No methylation was occurring on endogenous ribosomes (19) probably because the RNA was masked with protein. The ribose transmethylase activity described above appeared to be specific for mouse methyl deficient tRNA. The base transmethylase activity appeared to have less substrate specificity than the ribose transmethylase activity. The base transmethylases catalyzed the methylation of rRNA and *E. coli* tRNA in addition to mouse tRNA; however, base transmethylation occurs at a 10-fold elevated rate on methyl-deficient mouse tRNA when compared to normally methylated mouse tRNA. This suggests that there were base transmethylases in the extract which were specific for ethionine sensitive methylation sites in the methyl-deficient tRNA.

The apparent elevation of total transmethylase activity in Ehrlich ascites compared to mouse liver was greater when *E. coli* tRNA was used as a substrate than when methyl-deficient mouse tRNA was used (10-fold as compared to 2-fold); the enzymes which act upon *E. coli* tRNA were base transmethylases and represent the majority of the transmethylase activity in Ehr-
lich ascites cells. The base transmethylases which can use *E. coli* tRNA in vitro may recognize a tertiary structure common to most tRNAs and methylate a residue relative to that tertiary structure, as was previously discussed. The ability to recognize *E. coli* tRNA as a substrate in vitro represents a difference in the substrate specificities of the base and ribose transmethylases in Ehrlich ascites cell extracts. Most of the elevated tRNA transmethylase activity in Ehrlich ascites can be accounted for by base transmethylases. These base transmethylases may represent a subclass of transmethylases which recognize a tertiary structure on the tRNA instead of a particular nucleotide sequence.

The ratio of base to ribose transmethylase activities in Ehrlich ascites extracts was about 4:1 when methyl-deficient mouse tRNA was used as a substrate; therefore, it was impossible to study the ribose transmethylase activities in crude extracts without interference from the base transmethylases. One of the goals in this project was to purify the ribose transmethylase so that it could be studied without interference from the base transmethylases. When this purification was first attempted the strategy was to start with a
S100 fraction from Ehrlich ascites cells and to conduct filter binding assays after each fractionation to identify the ribose transmethylase activity. However, these assays required at least 24 hours to process so that it took about two weeks to purify the enzyme. The yield of ribose transmethylase in these first experiments was low (2-5%), and little purification of ribose transmethylase activity was achieved due to loss of enzyme activity. The first substantial purification of ribose from base transmethylase was achieved by isolating nuclei from homogenates of Ehrlich ascites cells. This nuclear fraction contained 84% of the ribose transmethylase assayable in the homogenate but only 5% of the base transmethylase activity. The transmethylase activities in this nuclear extract were fractionated using several techniques (section 15) and after each step the total transmethylase activity in each fraction was determined and the fraction having the highest total transmethylase activity was used in the next purification step. Ribose transmethylase activity was determined for the whole cell homogenate and the final preparation so that the overall purification of ribose transmethylase could be calculated. This procedure
yielded a 910-fold purified ribose transmethylase preparation in high yield with 10% base transmethyase contamination (figure 12). The ribose transmethylase activity in this preparation could be studied with nominal interference from the base transmethylase activity. The isolation of the nuclear fraction was an important step in the purification of ribose from base transmethylase in Ehrlich ascites homogenates. Initial experiments on the nuclear fraction determined that there was typically only 20% to 30% base transmethylase contamination.

Does base transmethylase activity in the purified ribose transmethylase (10% of the total transmethylase in the preparation) come about due to contaminating base transmethylases or to a minor base methylating activity of the ribose transmethylase? The answer to this question came in the final experiment in this project where Matrex Gel Green A chromatography was used as a purification technique by eluting the transmethylase activity with a salt gradient. This preparation had no base transmethylase activity suggesting that the base transmethylase activity in the previously purified preparations was due to contaminating enzymes.
The purified nuclear ribose transmethylase activity was optimized under the same conditions (pH 8.0, 0.1M KCl) as the ribose transmethylase assayed in the crude cell extracts. The nuclear ribose transmethylase activity accounted for a significant percentage of the total ribose transmethylase activity in the whole cell homogenate. These data argue that studies of this partially purified Ehrlich ascites nuclear ribose transmethylase were important in understanding the characteristics of the total ribose transmethylase activity in the cell. One cannot conclude from the 97% recovery of total ribose transmethylase activity in the purified enzyme (table 2) that all of the cellular ribose transmethylase activity was in the nucleus because the high recovery of activity may be affected by an activation of enzyme activity occurring during purification. [Note the apparent decrease of activity followed by an increase in activity after the DEAE-Sephadex step (table 2), a similar activation was observed in comparing the activities in S100 and DE-S100.]

Two months after the purification of the nuclear ribose transmethylase was initiated in this lab, Duane et.al.(33), published an abstract
reporting a ribose transmethylase in the nucleoli of Ehrlich ascites cells. There were data which suggested that the activity reported was not the same activity studied in our lab. The activity reported by the Florida group has an apparent molecular weight of 158,000 daltons as compared to an apparent molecular weight of 240,000 daltons for our transmethylase. They also reported that the ribose transmethylase was rRNA specific by using 18S and 28S nuclear rRNA as substrates. I tried methyl deficient nuclear RNA as a substrate for purified ribose transmethylase and found that it had only 25% the methyl accepting capacity as an equivalent mass of methyl-deficient tRNA (the nuclear RNA would be expected to have some tRNA). They also detected a low level of rRNA ribose transmethylation occurring at adjacent residues but I found only adjacent residues methylated by my purified transmethylase; perhaps both of these ribose transmethylases tend to methylate adjacent positions in vitro or perhaps their rRNA transmethylase preparation contains some contaminating transmethylase capable of such an activity. No tRNA ribose transmethylases have been purified (based on a computer search of the literature) although the
activities have been reported in crude extracts (5). Several tRNA base transmethylases have been purified and a summary of their characteristics is presented in table 4. There have been two reports (14, 34) of partially purified ribose transmethylases [(other than the work done by this lab and by Duane et al. (33)] and one of these enzyme preparations (14) had both base and ribose transmethylase activities, but it is not known whether both of the activities are due to a single transmethylase.

The determination of precise $K_m$ values for RNA and SAM was complicated by several factors. If undermethylated tRNA was used as a substrate, it would be difficult to determine what portion or species of the tRNA was acting as substrate. I have observed some variation in the apparent methyl accepting capacity of different preparations of methyl-deficient tRNA, for example the methyl-deficient tRNA preparation used in determining $K_m^{\text{tRNA}}$ had 75% the methyl accepting capacity of that used in determining $K_m^{\text{SAM}}$. The ideal substrate for the determination of precise $K_m^{\text{tRNA}}$ may be the transcript of a cloned tRNA gene.

The use of commercially produced SAM without further purification presents another problem
<table>
<thead>
<tr>
<th>Reference</th>
<th>Organism</th>
<th>Specificity</th>
<th>Molecular Weight (daltons x 10^-3)</th>
<th>kM_AM (uM)</th>
<th>kM_RNA (uM)</th>
<th>pH Optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>(5)</td>
<td>various organisms</td>
<td>tRNA base transmethylases</td>
<td>2.5 - 300</td>
<td>70 - 1.5</td>
<td>120 - 0.5</td>
<td>7 - 9</td>
</tr>
<tr>
<td>(31)</td>
<td>S. azureus</td>
<td>23S rRNA ribose transmethylase</td>
<td>38</td>
<td>150</td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>(14)</td>
<td>rat liver</td>
<td>nuclear rRNA 65% ribose, 35% base transmethylase</td>
<td>30</td>
<td>6.6</td>
<td></td>
<td>9.5</td>
</tr>
<tr>
<td>present study</td>
<td>Ehrlich Ascites Cells</td>
<td>tRNA ribose transmethylase</td>
<td>240</td>
<td>12.8</td>
<td>20</td>
<td>8.0</td>
</tr>
</tbody>
</table>
when determining $K_{m_{\text{SAM}}}$ as suggested in a recent report by Hoffmann(35). He determined that these commercial preparations are often contaminated with significant quantities (as much as 16% w/w) of S-adenosylhomocysteine (SAH), a potent inhibitor of methyltransferase reactions, and (R,S)-SAM, a biologically inactive stereoisomer of (S,S)-SAM. A sample of SAM from Boehringer-Mannheim (the brand used in this project) was analyzed (35) and found to be free of SAH but contaminated with 31% w/w (R,S)-SAM. If precise $K_{m_{\text{SAM}}}$ values are to be determined, commercially produced SAM should be fractionated to obtain pure (S,S) SAM. The apparent Km values in the present study were not presented as precise kinetic constants but rather as characteristic values under the conditions described. These apparent Km values will be useful in comparing values obtained under identical conditions using purified mouse liver ribose transmethylase.

Considerable data were generated in this study of Ehrlich ascites tumor and mouse liver transmethylases and methodologies were developed which will be useful in future research. Data generated in the comparative studies of transmethylase activities in mouse and Ehrlich ascites
homogenates suggested that much of the elevated transmethylase activity in the latter was due to base transmethylases which were highly active upon E. coli tRNA substrate. It was also shown that tRNA ribose transmethylases have greater substrate specificity than tRNA base transmethylases and were most active toward methyl deficient homologous tRNA substrates. A method for obtaining a purified (though not homogeneous) ribose transmethylase was developed and the purified transmethylase was partially characterized.

There were problems presented by the heterogeneous nature of the methyl-deficient tRNA and the SAM preparations used as substrates in this study and these problems must be dealt with before purified transmethylases can be characterized in depth. The purification of ribose transmethylase from mouse liver would facilitate comparative studies of purified ribose transmethylases from mouse liver and Ehrlich ascites. These studies would complement the comparative studies using crude homogenates accomplished in the present project.
REFERENCES


20. Wolfinbarger, L., personal communication. Old Dominion University, Norfolk, VA.


24. Higashi, K., Hanasaki, N., and Sakamato, Y (1978) Differences in Susceptibility to Sonication of Chromatins Containing Transcriptionally Active and Inactive Ribo-
somal Genes. Biochimica et Biophysica Acta 520: 612


27. Nazar, R., Owens, T., Sitz, T. and Busch, H. (1975) Maturation Pathway of Novikoff Ascites Hepatoma 5.8S Ribosomal Ribonucleic Acid. 250:2475


The vita has been removed from the scanned document