THE NUCLEOTIDE SEQUENCES OF FIVE LYSINE tRNAs FROM MURINE LYMPHOMA AND BALB/3T3

by

KIRK J. HAYENGA

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Approved by:

Charles Hedgcoth
Major Professor
Department of Biochemistry

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INTRODUCTION

The primary interest of this laboratory is in transfer ribonucleic acid, a group of molecules with molecular weights of about 23,000 and consisting of from 59 to 94 nucleotides. The most commonly known function of these molecules is their role in protein syntheses. tRNA accepts an amino acid and transfers it to the location of protein synthesis, the ribosome.

Besides the main function as a constituent in protein synthesis, tRNA plays other roles in the cell. It has been shown that in Staphylococcus epidermidis peptidoglycans are aminoacylated by an undermodified glycine tRNA (1, 2), which does not function in protein synthesis. In Escherichia coli lipopolysaccharides accept glycine from a glycyl-tRNA (3). Phosphatidylglycerol in other prokatyotes is known to be aminoacylated with lysine or alanine by transfer from tRNAs (4).

Transfer RNA has also been shown to be directly involved in the repression (5) and stimulation (6) of the transport of specific amino acids. Besides an involvement in transport, this nucleic acid also is known to control the attenuation of both the histidine (7, 8) and tryptophan (9) operons. It has been proposed that synthesis of other amino acids such as arginine (10), methionine (10), threonine (11), asparagine (12), valine (13), isoleucine (14), leucine (15), and glutamine (16) are regulated by tRNA in a similar fashion.

RNA tumor viruses package host cell tRNAs in a nonrandom manner (17), and it has been demonstrated that a specific tRNA, depending on the type of tumor virus, in conjunction with the viral enzyme reverse transcriptase, serves as a primer for DNA synthesis (18, 19).

Transfer RNA has demonstrated its prowess in the realm of enzyme regulation. Jacobson (20), working with Drosophila, has shown that a specific $tRNA^{Tyr}$ isoacceptor will interfere with the enzyme responsible for brown eye pigment. The enzyme, tryptophan oxygenase, seems to be inhibited by the presence of $tRNA_2^{Tyr}$. When the quantities of this isoacceptor are reduced, brown pigmentation returns to the eye.

Another example of a specific isoacceptor that may act as a regulator of cellular function is that of $tRNA_4^{Lys}$. Ortwerth's laboratory has shown that cells that are dividing or are capable of dividing possess levels of this isoacceptor over that of cells which are static (21, 22).

In the genetic code, three codons do not code for amino acids: UAG, UAA and UGA. They have been commonly termed nonsense codons because no amino acid normally is inserted within a protein in response to these codons. tRNAs in the tRNA pool normally do not decode these codons or at least not to a significant extent. These codons have also been called termination codons because translation is terminated in response to such codons in a message. If a nonsense codon appears in a coding region of a gene, an incomplete peptide will be formed. This type of event is called a nonsense mutation. Transfer RNA can undergo mutation in the anticodon giving it the ability to translate this codon and place an amino acid at the elongation site thereby suppressing the nonsense codon. This alteration is known as a nonsense suppressor mutation (23). Due to this modified tRNA a complete protein may now be synthesized, but it may differ from the normal protein by one amino acid at the site of suppression.

Many of the functions of tRNA concern a specific isoacceptor of a tRNA family. An isoacceptor is a member of a family of transfer RNA differing in structure or codon response but accepting the same amino acid.

We have been interested in the changes that occur in the lysine tRNA family between the normal state and the virus-transformed state of mammalian cells.

In 1974, Jacobson et al. (24) surveyed seven isoaccepting families looking for changes in the BD-cellulose[1] fractionation profiles between the normal mouse embryo cells and polyoma virus transformed fibroblasts in culture. The arginine, leucine, serine, and valine tRNA profiles demonstrated no variance in chromatographic profile. Histidine and tyrosine tRNAs showed no qualitative changes in profile but did exhibit differences in relative amounts of each isoacceptor. Lysine tRNAs displayed the most pronounced change in the isoacceptor profiles. The normal cells gave a profile containing two peaks: $tRNA_I^{Lys}$ and $tRNA_{II}^{Lys}$. When the transformed lysine tRNAs were chromatographed, the profile revealed three peaks. This finding suggested that there was a tRNA isoacceptor that was peculiar to the transformed state. Liu and Ortwerth (25) reported that $tRNA_I^{Lys}$ responded to the lysine codon AAG while $tRNA_{II}^{Lys}$ bound AAA. Through iodine oxidation (26) they showed that the anticodon in $tRNA_{II}^{Lys}$ probably contained a thiolated base (25).

With the advent of RPC columns (27, 28, 29) the separation and resolution of lysine tRNAs was greatly improved. This superior method produced two major peaks now designated as $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$. Three minor species were also present when RPC-5 (29) chromatography was applied (21). Ortwerth and Liu (21), using both normal and neoplastic cell sources, observed that one of these minor species, $tRNA_4^{Lys}$, was prevalent in cells which were dividing or that could be stimulated to divide. It was not found in cells that were not capable of dividing. This minor species was found to chromatograph between the two major species.

At the same time Ortwerth's laboratory found that $tRNA_4^{Lys}$ recognized the codon AAG and its ribosome binding was not affected by iodine oxidation (30). These findings indicated that $tRNA_4^{Lys}$ was probably very similar in structure to $tRNA_2^{Lys}$.

Juaraz et al. (31) reported the separation of seven lysine isoaccepting tRNAs from polyoma virus by RPC-5 chromatography. One of the two new tRNAs, $tRNA_6^{Lys}$, was specific for this type of cell. The other new peak, $tRNA_{5a}^{Lys}$, chromatographed with $tRNA_5^{Lys}$ with very little resolution.

Katze (32) reported that there are as many as seven lysine tRNA isoacceptors in SVT2 cells in culture.

Possible functions of the various isoacceptors poses a very interesting problem. Codon binding studies were performed using isoacceptors 1, 2, 4, 5, 5a, and 6 (33). It was found that peaks 1, 2 and 4 recognize the codon AAG while the late eluting peaks 5, 5a, and 6 respond to AAA. Utilization of each species was determined by use of a wheat germ protein synthesis system in conjunction with tobacco mosaic virus mRNA. In this study, $tRNA_6^{Lys}$ participated only 14-24% as effectively as the other isoacceptors (33). These experiments also pointed out that tRNAs binding the AAA codon lost acceptor capacity when exposed to iodine oxidation, indicating the presence of a thiolated nucleotide in the anticodon region of these tRNAs.

Hatfield et al. (34) recently reported that only 25% of $tRNA_4^{Lys}$ from rabbit liver is used in the presence of globin mRNA, whereas 60% and 32% of $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$, respectively, are used.

Lysine isoacceptors 5a and 5, from mouse LM cells, are both sensitive to iodine oxidation and they respond to the codon AAA (22). In mouse L cells, the relative amounts of each tRNA depends upon the nutritive state of the

cells. These results lend support to the idea that $tRNA_{5a}^{Lys}$ is a modified form of $tRNA_5^{Lys}$ (22). $tRNA_{5a}^{Lys}$ has also been shown to be the major isoacceptor aminoacylated by cytoplasmic synthetase in mouse liver mitochondria (35). When $tRNA_5^{Lys}$ was exposed to iodine oxidation not all of the tRNA under that peak irreversibly lost its potential to accept lysine. The surviving species is known as $tRNA_{5b}^{Lys}$ (36). This isoacceptor recognizes the codon AAG, but only after the effects of iodine oxidation are reversed. These data suggest that this species is closely related to $tRNA_1^{Lys}$, $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ but chromatographs with $tRNA_5^{Lys}$ due to the presence of a thiolated nucleotide in its structure (36).

Raba et al. (37) have sequenced $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$ from rabbit liver and $tRNA_4^{Lys}$ from SV-40 transformed mouse fibroblasts. Their findings do show a close relationship between $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ as earlier results suggest and that $tRNA_5^{Lys}$ does contain a thiolated nucleotide in the anti-codon region. Their results would be stronger if a single tissue source had been used as a source of all three tRNAs sequenced.

Transfer RNA_6^{Lys} appears to be associated only with the transformed state or cells that have been stressed (38). Thomas showed that this species is associated with the mitochondria (38). This finding has been supported by experiments showing that $tRNA_6^{Lys}$ aminoacylates in vivo with exogenously supplied [^{3}H]-lysine at a slower rate than does the bulk of the lysine isoacceptors, suggesting compartmentation (39).

Ortwerth and Liu (40) have recently proposed a complex model for the interrelationship of the lysine tRNA family. To provide a definitive base for this theory, sequencing data must be compiled on all lysine isoacceptors.

The theme of this dissertation is the determination of the nucleotide sequences of selected members of the lysine tRNA family from a transformed

cell line. These data will provide useful information in determining the metabolic relationship of the lysine tRNA family.

In 1965 Robert Holley reported the sequence of the first nucleic acid (41). The work involved a time consuming and tedious process. The RNA was hydrolyzed by nucleases to give a set of smaller degradation products each of which required additional enzyme hydrolysis and fractionation by column chromatography on DEAE-cellulose in a denaturing medium.

Sanger and coworkers (42) also applied the action of ribonucleases but fragments of RNA were then separated by two-dimensional electrophoresis. Each oligonucleotide produced was further digested by ribonucleases and identified by their migration pattern after electrophoresis. This method was useful only if relatively large quantities of RNA were available and if it could be labeled in vivo with ^{32}P to a high specific activity.

In vitro methods for ^{32}P-labeling have been developed that now permit the sequencing of very small amounts of RNA. The 3' end of a tRNA molecule can be labeled either by adding a radioactive nucleotide such as [5'-^{32}P]pCp with T_4 RNA ligase (43) or replacing the terminal adenosine with a radio-labeled adenosine using nucleotidyl transferase (44). The labeling of the 5' end requires the production of a 5' hydroxyl group. This can be accomplished by removing the 5' phosphate with alkaline phosphatase. The 5' end may be labeled using [^{32}P]ATP and polynucleotide kinase. Thus, RNAs labeled in vitro now can be subjected to a sequencing method similar to that of Sanger et al. (42). This procedure starts with RNase T_1 digestion to obtain oligonucleotides followed by labeling the RNA with [γ^{32}-P]ATP and polynucleotide kinase after removal of the 5' phosphate with alkaline phosphatase. The fragments can be separated by either two-dimensional

electrophoresis or by a combination of electrophoresis and ion exchange chromatography (homochromatography). These oligonucleotides then are sequenced after partial hydrolysis with either nuclease P_1 or snake venom phosphodiesterase. A nested set of fragments differing by one nucleotide is produced by withdrawing an aliquot from the reaction mixture at specified times. This set is fractionated by a two-dimensional system consisting of electrophoresis on cellulose acetate at pH 3.5, transferring to a DEAE-cellulose thin layer plate and chromatographing. The sequence is determined by the mobility shift of each fragment relative to the preceding fragment.

There are a couple of drawbacks to this procedure. In order to identify the modified nucleotides found in tRNA, one must be familiar with the mobility shifts of these minor bases. This method can only sequence a limited number of nucleotides during one two-dimensional run.

Recently, rapid sequencing techniques have become available which can yield an entire tRNA sequence without repetitive applications of a single method.

Peattie (45) developed a chemical sequencing method that is very similar to the chemical method of DNA sequencing designed by Maxam and Gilbert (46). This method is based on four base-specific chemical reactions, one reaction for each of the four major nucleotides. The RNA introduced to the reaction has been labeled at the 3' termini by the method of Bruce and Uhlenbeck (47) involving [5'-^{32}P]pCp and T_4 RNA ligase. Once the base has been modified by the specific reaction, it is subject to strand scission. The breaking of the phosphate-ribose backbone is accomplished by an aniline-catalyzed β-elimination.

The conditions of the reactions are set up so that the nucleotides are hit at random, and by the termination of the reaction every nucleotide has

been hit within the set population of tRNA with each molecule suffering a single hit. In conjunction with the strand scission this produces a nested set of fragments all differing in length by one nucleotide. These then can be separated by polyacrylamide gel electrophoresis where each separate reaction is run in an adjacent lane. The sequence can be read directly from an autoradiogram of the gel.

This method will only produce the sequence for the major nucleotides of tRNA. Where a minor nucleotide exists, a gap will be present.

Another recently developed method that gives the major nucleotide sequence is "enzyme sequencing" (48, 49). This method is similar to chemical sequencing in that the major objective is to produce a nested set of ^{32}P-labeled RNA fragments. This is accomplished by breaking the RNA at random sites under limited conditions of ribonuclease hydrolysis with several ribonucleases of differing specificities. Each of the reactions can be loaded on a polyacrylamide gel in adjacent lanes and the fragments resolved. As in chemical sequencing the sequence can be read directly from an autoradiogram of the gel.

Both the chemical and enzymatic sequencing techniques are rapid and use as little as 1 μg of tRNA to obtain a complete sequence. The enzymatic sequencing method does not cleave RNA significantly in regions that are double stranded. This is one of the disadvantages of this method. The biggest drawback for applying these techniques to tRNA sequencing is that modified nucleotides are not identified as they are in the mobility shift technique.

Stanley and Vassilenko (50) developed a procedure that produces a complete nested set of fragments by formamide hydrolysis. RNA is dissolved

in formamide and heated to between 90 to 100° for a specified period to give hydrolysis products derived from single hit kinetics providing breaks at every phosphodiester linkage but with only one hit per molecule. This hydrolysis produces a free 5' hydroxyl group at the site of breakage. The 5' end of the fragment is labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase.

The nested set of fragments is resolved by polyacrylamide gel electrophoresis in a denaturing medium, commonly 7 M urea.

After location of each band by autoradiography, each band is excised and the fragment is eluted from the gel piece. The 5' labeled nucleotide is chopped from the molecule through alkaline hydrolysis. These nucleotides are then separated by electrophoresis on paper using a standard pH 3.5 buffer. The nucleotides are identified by their mobility as compared to standards.

Gupta and Randerath (51) have applied limited alkaline hydrolysis to another sequencing method. This method is based upon attainment of a nested set of fragments from hydrolysis in water. The 5' hydroxyl created by the hydrolysis is labeled as in the Stanley-Vassilenko method and labeled fragments are separated by electrophoresis on a denaturing polyacrylamide gel. The fragments are then transferred to a thin layer PEI-cellulose plate by a blot or contact transfer method.

The thin-layer plate is autoradiographed to locate bands and the 5'-labeled nucleotide of each band is freed by applying RNase T_2 directly to the thin layer plate. The lane of bands is cut longitudinally to provide duplicate portions and nucleotides of bands of each strip are contact transferred in a single step to another PEI-cellulose plate for

chromatography. The resulting thin layer sheets are chromatographed, one in each of two systems, an ammonium sulfate system and an ammonium formate system. The sheets are autoradiographed to locate nucleotides, which are identified by mobilities. This method identifies directly many of the minor nucleotides found in tRNA. In addition, there are two-dimensional thin layer chromatography systems to aid in identification (52, 53).

As stated previously, the main theme of this thesis is the characterization and structural determination of tRNAs from a transformed cell source. It is crucial that the structures of tRNAs from transformed tissue sources be elucidated so that comparisons to tRNA from normal sources can be made. Structural differences most likely hold the key to tRNA functional differences between the two sources. Due to extensive nucleotide modification in tRNA, it is thought that alterations in modification are responsible for changes in functional ability. Determination of nucleotide sequences of tRNAs from neoplastic tissue is a relatively new area. At this time the library of known tRNA sequences from transformed tissues is very limited (37, 54-62). The work reported here has succeeded in expanding this library of known sequences.

EXPERIMENTAL PROCEDURES

Preparation of [γ-^{32}P]ATP and [5'-^{32}P]pCp

[γ-^{32}P]ATP and [5'-^{32}P]pCp were prepared according to the method of Walseth and Johnson (63). [γ-^{32}P]ATP is produced by substrate-level phosphorylation employing glycolytic enzymes and intermediates. A reaction mixture is made containing [^{32}P]PO_4^{-3}, ADP and the necessary glycolytic intermediates. To initiate the reaction a suspension of the appropriate glycolytic enzymes is added. The reaction course is followed by observing the difference between Norit A adsorbable and Norit A unadsorbable counts. When the unadsorbable counts are 5% of the adsorbable counts, the reaction is terminated.

[5'-^{32}P]pCp production begins with [γ-^{32}P]ATP. In the presence of 3'-CMP and polynucleotide kinase the radioactive phosphate from [γ-^{32}P]ATP is transferred to the 5' position on 3'-CMP. The quantitation also depends upon the comparison of Norit A adsorbable and unadsorbable counts.

Storage of enzymes. The enzyme mixture was prepared as follows: 100 µl of 2 mg/ml L-α-glycerolphosphate dehydrogenase, 1 µl of 2 mg/ml triose phosphate dehydrogenase, 20 µl of 10 mg/ml glyceraldehyde-3-phosphate dehydrogenase, 2 µl of 10 mg/ml 3-phosphoglycerate kinase, 20 µl of 5 mg/ml lactate dehydrogenase. This mixture can be stored at 4°. The enzymes were purchased from Boehringer-Mannheim.

Preparation of enzymes for reaction mixture. A sample, 15 µl, of the stored enzyme mixture was centrifuged for 10 min at 12,000xg in an Eppendorf 5412 centrifuge. The pellet was dissolved in 15 µl of 50 mM Tris-HCl, pH 9.0. Five microliters of the redissolved enzyme mixture was added to 65 µl

of a solution containing 7 µl of 500 mM Tris-HCl, pH 9.0, and 2.7 µl of 130 mM dithiothreitol.

Reaction mixture. The reaction was done in a 1.5 ml Eppendorf polypropylene conical tube containing the following: 0.6 µl of 1 M magnesium acetate, 2.5 µl of 36 mM spermine (Sigma), 2.5 µl of 130 mM dithiothreitol (P-L Biochemicals), L-α-glycerolphosphate (Boehringer Mannheim), 2.5 µl of 1 mM ADP (Sigma), 2.5 µl of 10 mM β-NAD^+ (Boehringer Mannheim), 2.5 µl of 20 mM sodium pyruvate (Sigma), 2.92 µl of 1 M Tris-HCl, pH 9.0, and 250 pmol of $[^{32}P]PO_4^{-3}$ (New England Nuclear), 25 mCi/200 µl. The solution was adjusted to 45 µl with sterile deionized water. The reaction was initiated with 5 µl of the enzyme mixture and was allowed to proceed at room temperature (24°).

The reaction may be run in the pH range of 7.5-9.0. The $[^{32}P]PO_4^{-3}$ was lyophylized if the volume added would bring the total reaction volume to over 50 µl. All of the solutions were either autoclaved or prepared with sterile deionized water.

Quantitation of the reaction. An inch long thread of surgical silk was placed in the reaction vessel so that the end of the thread touched the bottom of the tube. The thread was removed and placed in a 1.5 ml conical tube containing 1 ml of 50 mM KH_2PO_4. A sample, 25 µl, of this solution was pulled and placed in a separate conical tube. Norit A, 50-100 mg, was added to the remaining KH_2PO_4. After mixing and centrifugation, 25 µl of the supernatant solution was transferred to a separate conical tube. The amount of radioactivity in the 25 µl samples collected before and after treatment of the diluted "thread aliquot" with Norit A was determined periodically by Cerenkov counting over a period of 30 min. The radioactivity not

adsorbed to Norit A represents unreacted $[^{32}P]PO_4^{-3}$. When the cpm not adsorbed to Norit A were 5% of the total cpm, the reaction was terminated. The reaction usually was essentially complete in 30 min. (The surgical silk was graciously furnished by Lafene Health Center, KSU.)

Termination. The reaction vessel was placed in boiling water for 12 min. If the protein in the reaction vessel is not deactivated, the $[\gamma\text{-}^{32}P]$ATP will slowly undergo hydrolysis (63).

Production of $[5'\text{-}^{32}P]$pCp

The $[\gamma\text{-}^{32}P]$ATP produced in the preceding step is used in conjunction with polynucleotide kinase to produce $[5'\text{-}^{32}P]$pCp (63).

Reaction mixture. After terminating the reactions producing $[\gamma\text{-}^{32}P]$ATP, 10 µl of 9.28 mM 3'-CMP and 1.5 µl (5900 units/ml) of polynucleotide kinase (New England Nuclear) were added. The reaction was allowed to proceed overnight at room temperature (24°). All solutions were made with sterile deionized water.

Quantitation of the reaction. Two inch-long threads of surgical silk were placed in the reaction vessel so that the ends of the thread touched the bottom of the conical tubes. One string was placed in a tube (tube A) containing 500 µl of 50 mM Tris-HCl, pH 8, 20 µg of hexokinase (460 units/mg) and 4 mM glucose. The other string was placed in a conical tube (tube B) containing 500 µl of 50 mM Tris-HCl, pH 8.0. Both conical tubes were incubated for 15 min at room temperature. Then, to each tube was added 500 µl of 50 mM KH_2PO_4 to terminate the reactions. A 25 µl sample was transferred from both tubes A and B to separate conical tubes. Then, to both tubes A and B 50-100 mg of Norit A was added. After mixing and centrifugation, another 25 µl of sample was transferred from both tubes

A and B to separate conical tubes. The amount of radioactivity in the four 25 µl samples was determined by Cerenkov counting.

The difference in counts between the two A tubes gives the amount of $[^{32}P]PO_4^{-3}$ left in solution. The difference in counts between the two B tubes gives the amount of $[^{32}P]PO_4^{-3}$ and $[\gamma\text{-}^{32}P]ATP$ left in solution. When the number of unadsorbed counts in the A tubes equals the unadsorbed counts in the B tubes, the reaction is complete.

Purification of Lysine tRNAs

Lysine isoaccepting tRNAs were obtained from either murine lymphoma (L5178Y) cells or stressed Balb/3T3 cells in culture. The Balb/3T3 cells were stressed by lowering the level of calf serum from 10% to 1%.

The tRNAs were purified by Dr. B. J. Ortwerth according to the method of Wittig et al.(64). tRNA was obtained by phenol extraction of the cells. Lysine isoacceptors were fractioned on RPC-5 and rechromatographed on RPC-5. Each isoacceptor was aminoacylated with ^{14}C-lysine and chromatographed on DEAE-cellulose. A naphthoxyacetyl derivative was formed probably involving both the alpha and epsilon amino groups of lysyl-tRNAs. Each derivatized tRNA then was chromatographed on BD-cellulose to achieve essentially pure isoacceptors of lysine.

If the sequencing technique applied required labeling of the tRNA on the 3' terminus, the isoacceptor was stripped to remove the naphthoxyacetyl lysine moiety.

3'-Labeling of Lysine tRNA with $[5'\text{-}^{32}P]pCp$

Labeling of the 3' terminus for chemical and enzymatic sequencing procedures was done by modifying the conditions used by Peattie (45).

A 30 pmol sample of [5'-^{32}P]pCp either commercially prepared (New England Nuclear, 2.9 μCi/pmol) or prepared in the laboratory (9 μCi/pmol) was lyophilized to dryness in a 1.5 ml conical tube. Once [5'-^{32}P]pCp is lyophilized, it should be used within 30 min to avoid autoradiolysis. To the dried [5'-^{32}P]pCp the following items were added: 30 pmol of lysine tRNA, 2.5 μl of a solution containing 50 mM Hepes, 15 mM magnesium acetate and 0.01 mg/ml bovine serum albumin (BRL, nuclease free), 1 μl of 33 mM dithiothreitol, 1 μl of 222 pmol/μl ATP, 1 μl of dimethylsulfoxide, 0.5 μl of T_4 RNA ligase (4300 units/ml P-L Biochemicals), and sufficient deionized water to bring the final volume to 10 μl. The reaction was incubated overnight on ice. Then, 3 μl of 1.5 M sodium acetate and 1 μg of carrier tRNA were added. tRNA was precipitated with 40 μl of ethanol (-20°). The solution was thoroughly mixed by inverting the tube several times and placed in a -70° dry ice/ethanol bath for 10 min. After centrifugation for 5 min at 12,000xg the pellet was washed with 20 μl of ethanol (-20°) and dried in vacuo. The pellet was dissolved in 3 μl of formamide loading solution: 20 μl of 25 mg/ml each of xylene cyanol FF and bromophenol blue, 100 μl of 900 mM Tris-HCl, pH 8.3, with 10 mM Na_2EDTA, 80 μl of sterile deionized water and 800 μl of deionized formamide. This solution was mixed thoroughly and stored at -20° until applied to a purification gel.

Preparation of Unlabeled tRNA for Gel Purification

Chromatographically purified $tRNA^{Lys}$, 0.05 A_{260} units, was lyophilized to dryness and dissolved in 3 μl of formamide loading solution with vortexing. This solution was stored at -20° until applied to a purification gel.

Purification of Lysine tRNAs by Polyacrylamide Gel Electrophoresis

Both labeled and unlabeled lysine tRNAs were purified by electrophoresis in a 30 cm by 15.5 cm by 0.4 mm thickness gel and then used immediately in a sequencing procedure.

Preparation of 10% gel. The gel solution was prepared in a 50-ml graduated cylinder by adding 12.5 ml of 40% acrylamide/bisacrylamide (19:1) (BioRad), 5 ml of 900 mM Tris-borate, pH 8.3, with 10 mM EDTA (Sigma), and 21 g of urea (dried, Ultrapure, Schwarz/Mann Inc.), and stirring the mixture at 37° until the urea dissolved. The final volume was adjusted to 50 ml with distilled water. The solution was filtered through a Millipore membrane and deaerated. The gel solution was split into three parts: a 5 ml plug, a 5 ml patch and the remainder in a 50 ml beaker. A plug was prepared by adding 25 µl of 10% ammonium persulfate to the plug solution and swirling without introducing air into the solution. After adding 1 µl of TEMED and swirling cautiously, the plug was poured into an assembled plate system set at a 45° angle with the bottom sealed with plasticine. After about 10 min, 0.2 ml of 10% ammonium persulfate was added to the major part of the gel solution. After swirling the solution, 10 µl of TEMED was added. The solution was mixed by swirling and poured into the plate assembly which then was lowered to a horizontal position, and the well former was inserted. Patching of the polymerized gel was done if necessary. The gel aged for 2 hr and was preelectrophoresed for at least 2 hr at 350 v.

Electrophoresis. The tRNA sample was heated at 37° for 3 min, quickly-chilled on ice, and loaded into a "blown-out" well with a finely drawn-out capillary tube. Electrophoresis was done at 350 v until xylene cyanol FF reached 25 to 28 cm. The usual running time was 16 to 18 hr.

Detection and recovery of tRNA. Labeled tRNA was detected by autoradiography and unlabeled tRNA was detected by staining the tRNA band. For autoradiography, the gel was left attached to one of the glass plates, spotted in the corners with a ^{32}P-labeled compound, covered with clear polyethylene sheet (like Glad Wrap), and exposed to x-ray film (Kodak X-Omat AR-5) for a few seconds to 2 min. The tRNA band was excised with a flamed scalpel and placed into an autoclaved, siliconized conical tube.

For unlabeled tRNA, the gel, still attached to one glass plate, was stained for 2 min in 200 ml of 0.4% methylene blue. The gel was destained with seven changes of distilled water for 1 min each. The tRNA band was excised as above.

tRNA was eluted from gel pieces with a volume of elution buffer (50 mM Tris-HCl, pH 7.5, 400 mM NaCl, and 20 µl/ml of 25% SDS) ten times that of the gel pieces for 2.5 hr at 37°. The gel pieces were removed with flamed forceps, and tRNA was precipitated with 3 volumes of ethanol (-20°), chilled at -70°, and then kept from 2 hr to overnight at -20°. tRNA was recovered by centrifugation for 5 min at 12,000xg.

Enzymatic Sequencing of Purified $tRNA_6^{Lys}$

The sequence of major nucleotides of $tRNA_6^{Lys}$ was elucidated by an enzymatic sequencing method based on the procedure of Donis-Keller et al. (48) and Lockhard et al. (65). The protocol was provided by B. Roe (66).

The ribonucleases and their nucleotide specificities are as follows: RNase T_1 is guanine specific, RNase U_2 is adenine specific, RNase from Bacillus cereus is specific for pyrimidines, RNase Phy M from Physarum polycephalum cleaves at uracil and adenine, and RNase Cl_3 from chicken liver hydrolyzes the phosphodiester linkage on the 3' side of cytidines.

The T_1, U_2, B. cereus and Phy M RNases, all from P-L Biochemicals, were dissolved in 20 µl of sterile water (10 units/µl). RNase Cl_3 (BRL) was used undiluted.

A [5'-^{32}P]pCp-labeled tRNA was divided into seven aliquots in 1.5 ml conical tubes, each containing at least 14,000 cpm (Cerenkov counts), and each sample was lyophilized to dryness. To each of the seven tubes 5 µl of the appropriate reaction mixture was added. The RNase T_1 and Phy M reaction mixtures contained 100 µl of 10 M urea (ultrapure), 45 µl of sterile deionized water, 3 µl of 1 M sodium citrate, pH 5.0, 1.5 µl of 0.1 M EDTA, and 2 µl of 2% xylene cyanol and bromophenol blue. The reaction mixture for RNase U_2 was the same as T_1 except the sodium citrate was pH 3.5. RNase B. cereus reaction mixture was the same as T_1 and Phy M with the exception that 100 µl of water was substituted for the urea. The reaction mixture for RNase Cl_3 consisted of 70 µl of 10 M urea, 18 µl of water, 10 µl of 0.1 M phosphate buffer, pH 6.7, and 2 µl of 2% xylene cyanol and bromophenol blue. Five microliters of formamide loading solution was added directly to the residue for the control conical. The formamide hydrolysis reaction solution was made up of 1 ml of deionized formamide, 1 µl of 1 M magnesium acetate, and 20 µl of 2% xylene cyanol and bromophenol blue. Each tube was vortexed, heated for 5 min at 55° and immediately chilled on ice. Each enzyme, with the exception of RNases Phy M and Cl_3, was diluted 1/10, and 1 µl of each was added to the respective tube on ice and mixed gently. The enzymatic reactions and control were incubated 15 min at 55°. The tube with formamide was heated at 95° for 12 min. Immediately after incubation, each conical tube was chilled on ice. To the RNase Phy M reaction, 3.3 µl of 10 M urea was added. Each tube remained on ice or was stored at -20°.

Chemical Sequencing of Lysine tRNAs

A chemical method, like enzymatic sequencing, gives the sequence of the major nucleotides. The chemical sequencing method of Peattie (45) was used. The nested set was produced by modifying the bases with four separate base-specific chemical reactions under conditions providing essentially single-hit kinetics. Each of four aliquots of a [5'-^{32}P]pCp-labeled tRNA is exposed to one of the four specific chemical reactions. Following the modification and subsequent loss of the base, strand scission was accomplished through aniline catalyzed β-elimination.

The guanine nucleotides were methylated at the N-7 position with dimethylsulfate. Methylation was followed by a reduction step involving sodium borohydride.

Diethylpyrocarbonate reacts with adenosine at the N-7 position. This involves a carbethoxylation of the base nitrogen. The result of this reaction is a ring opening of the imidazole ring. The diethylpyrocarbonate will attack guanine, but at a rate seven times slower than reactions with adenosine.

The 5-6 double bond of uridine is subject to a nucleophilic addition with unprotonated hydrazine. Cytidine also reacts with hydrazine in the same manner. The conditions for this reaction must be set up so that the hydrazinolysis is specific for cytidine. Anhydrous hydrazine in the presence of 3 M sodium chloride is specific for cytidine residues. When a tRNA molecule possesses a 7-methylguanosine, this reaction must be modified (83). Hydrazine containing 3 M sodium chloride will specifically cleave at 7-methylguanosine leaving no fragment longer than this position. The modification involves the methylation of cytidines followed by an

aqueous hydrazine treatment. Under these conditions the cytidine nucleotides will be specifically hit.

After each reaction was completed the RNA phosphodiester backbone was cleaved by an aniline catalyzed β-elimination.

When strand scission was completed, the samples were lyophilized and dissolved in 7 µl of formamide loading solution. The tubes were heated at 90° for 30 sec, chilled on ice and the solutions were loaded directly onto a 12% denaturing sequencing gel.

The Production of Formamide Hydrolysis Fragments for the Sequencing of tRNAs

Formamide hydrolysis was based on the procedure of Stanley and Vassilenko (50). A dried purified tRNA, approximately 0.05 A_{260} units, was dissolved with vortexing in 10 µl of deionized formamide. The solution was transferred to a siliconized capillary tube and the ends were flame sealed. The capillary was heated in a water bath at 95-92° for 4 min. The sample was transferred to a conical tube and 10 µl of 1 M sodium chloride was added. Fragments were precipitated by adding 60 µl of ethanol (-20°) and chilling at -70° for 5 min. Fragments were collected by centrifugation for 5 min at 12,000xg, washed with 40 µl of ethanol (20°), pelleted by centrifugation at 12,000xg and dried *in vacuo*.

Labeling conditions were modified from those of Gupta and Randerath (51). The dried fragments were dissolved with vortexing in 5 µl of a reaction mixture: 50 pmol [γ-^{32}P]ATP, 1 µl of 0.4 M Tris-HCl, pH 8.7, 1.3 µl of 0.1 M magnesium acetate, 0.8 µl of 130 mM dithiothreitol, 0.8 µl of ATP (226 pmol/µl), and 6 µl of sterile deionized water. To this was added 0.5 µl of 0.1 mg/ml bovine serum albumin (BRL, nuclease free), and 0.5 µl of

polynucleotide kinase (5000 units/ml, New England Nuclear). The solution was incubated at 37° for 25 min. Incubation was terminated by the addition of 5 µl of 1 M ammonium acetate and chilling. Carrier tRNA, 10 µg, was added and immediately followed with 40 µl of ethanol (-20°). The solution was mixed by inverting the tube several times, chilled at -70° for 5 min and centrifuged at 12,000xg for 5 min. The resulting pellet was dried in vacuo and dissolved in 7 µl of formamide loading solution. The sample was stored at -20° until ready for use. The sample was heated at 55° for 3 min and immediately chilled before each addition to the gel. The samples were either applied to a 12% or a 20% denaturing polyacrylamide gel. If both gels were used the volume of loading solution was increased by 6 µl.

Production of Sequencing Ladder by Polyacrylamide Gel Electrophoresis

The nested sets of fragments produced by chemical or enzymatic means or formamide hydrolysis were separated on either a 12% or a 20% polyacrylamide denaturing gel. Selection of gel percentage depended upon the size of fragments to be resolved. The 20% gel resolved smaller fragments while the 12% gel proved to be superior in separating the larger fragments.

Preparation of 12% (20%) gel. The gel solution was prepared in a 100 ml graduated cylinder by adding 30 ml (50 ml) of 40% acrylamide/bisacrylamide (19:1) (BioRad), 5 ml of 900 mM Tris-borate, pH 8.3, with 10 mM EDTA (Sigma), and 21 g of urea (dried, Ultrapure, Schwartz/Mann Inc.), and stirring the mixture at 37° until the urea dissolved. The final volume was adjusted to 100 ml with distilled water. The solution was filtered through a Millipore membrane and deaerated. The gel solution was split into three parts: a 5 ml plug, a 5 ml patch and the remainder in a 100 ml beaker. A plug was prepared by adding 25 µl of 10% ammonium persulfate to the plug

solution and swirling without introducing air into the solution. After adding 1 µl of TEMED and swirling cautiously, the plug was poured into an assembled plate system set at a 45° angle with the bottom sealed with a spacer. After about 10 min, 0.45 ml of 10% ammonium persulfate was added to the major part of the gel solution. After swirling the solution, 20 µl of TEMED was added. The solution was mixed by swirling and poured into the plate assembly which then was lowered to a horizontal position and the well former was inserted. Patching of the polymerized gel was done if necessary. The gel was aged for 12 hr and preelectrophoresed for a minimum of 8 hr.

Electrophoresis. Each tRNA sample was heated according to instructions under each procedure. Electrophoresis was done using 90 mM Tris-borate, pH 8.3, and 1 mM EDTA as buffer. The gel temperature was maintained at 48 to 52°. Run time was dependent upon the region to be resolved. Each reaction was loaded at one or two different times after initiating the run to improve resolution of different parts of the fragment ladders.

Autoradiography. The gel sandwich was separated leaving the gel on one of the plates. The gel was covered with a clear polyethylene film and autoradiographed at -70° with Kodak X-Omat AR-5 x-ray film placed between the gel and a Dupont Cronex Lighting-Plus ZH intensifying screen. The autoradiography time, 6-24 hr, depended upon the activity of the sample load. The sequence was read directly from the autoradiogram for procedures involving enzymatic or chemical means. Gels involving formamide hydrolysis ladders were marked with radioactive dye spots and further processed.

Determining the Sequence of tRNAs from Formamide Sequencing Ladders

Recovery of fragments from the ladder. To identify the 5'-labeled

nucleotides, bands must be excised and fragments eluted. The autoradiogram was positioned with the gel so that the radioactive marker spots aligned with the marker spots of the sequencing lane. Each band was excised using a flamed scalpel and placed in a conical tube. To each tube was added 300 µl of elution buffer (50 mM Tris-HCl, pH 7.5, 400 mM sodium chloride and 20 µl/ml 25% SDS). The tube was maintained for 6 hr or overnight at 37°. Eluted fragments were precipitated by adding 10 µg of carrier tRNA and 900 µl of ethanol (-20°), mixing well, and chilling at -70° for at least 10 min. After centrifugation for 5 min the pellet was dried *in vacuo*. The pellet was dissolved in 10 µl of 10 mM ammonium acetate containing 0.015 units/µl of RNase T_2, and incubated 6 hr or overnight at 37° after which the solution was evaporated *in vacuo*. The residue was dissolved in 4 µl of sterile deionized water, and the solution was stored at -20°.

Identification of 5' labeled nucleotides and reading of the sequence. An aliquot from each tube was spotted on two separate predeveloped PEI-cellulose TLC plates. The aliquot contained at least 250 cpm (Cerenkov counts). Each plate was spotted with 17 samples. One plate was chromatographed in 0.55 M ammonium sulfate while the other plate was soaked in 0.1 M ammonium formate, pH 3.5, dried, and chromatographed in 1.75 M ammonium formate (51). A 20 cm x 7.5 cm wick was attached to each plate. The solvent fronts were allowed to run 1.5 to 2.5 cm onto the wick. The wicks were removed, and each TLC plate was spotted with radioactive ink and autoradiographed.

Two-dimensional identification of nucleotides. When the identification of a nucleotide required further clarification, the sample was subjected to two-dimensional chromatography. The procedures used were those of Silberklang *et al.* (52) and Nishimura (53).

The remaining 2 µl from the above procedure was incubated overnight at 33° with 2 µl of RNase P_1 (1 µg/µl). An aliquot, containing at least 100 cpm, was spotted on a 10 cm x 10 cm cellulose TLC plate which had been prespotted with 0.5 µl of a mixture containing pNs (pΨ, pU, pT, pC, pG, pA, and pUm) of a concentration of 10 nmol/µl each.

A chromatogram was developed in one of two two-dimensional solvent systems. Solvent system 1 consisted of the following solvents: first dimension, i-butyric acid/water/ammonium hydroxide, 132:66:2; second dimension, 0.1 M sodium phosphate, pH 6.8/ammonium sulfate/n-propanol, 100:60:2. Solvent system 2 consisted of: first dimension, identical to the first dimension in system 1; second dimension, i-propanol/HCl/water, 70:15:15. The chromatograms were allowed to dry for 24 hr between the first and second chromatographic ascents. This was done to completely rid the chromatogram of i-butyric acid. Each chromatogram was then spotted with ^{14}C-ink and autoradiographed for a period of 3-5 days.

Determination of Total Nucleoside Composition

The total nucleoside composition was quantitatively and qualitatively determined by the procedure of Randerath et al. (69).

This procedure involves enzymatic digestion of tRNAs to nucleosides, periodate oxidation of the linkage between the 2' and 3' carbons of the ribose, reduction of the resulting dialdehydes to alcohols with $[^3H]KBH_4$, and two-dimensional chromatography of the products on thin layers of cellulose. Nucleosides were located by autoradiography and eluted, and the amount of radioactivity in each was determined.

RESULTS

Purification of tRNAs

Isoacceptors of lysine tRNA, purified chromatographically as described in Experimental Procedures, were supplied by Dr. Ortwerth. I further purified these tRNAs by either one-dimensional or two-dimensional polyacrylamide gel electrophoresis.

Transfer RNA_2^{Lys} and $tRNA_4^{Lys}$ from murine lymphoma cells were both purified by one-dimensional electrophoresis (Fig. 1). Each lane of the gel represents 30 pmol of [5'-^{32}P]pCp-labeled tRNA.

$tRNA_2^{Lys}$ showed two distinct autoradiographic bands and a lighter band 1 cm above the heavier labeled top band. The darkest band represents the major species of RPC-5 peak 2; the lighter of the two distinct bands is the minor species. Transfer RNA_4^{Lys} gave the same pattern as $tRNA_2^{Lys}$ with less resolution but is labeled to a higher specific activity. This higher activity made it possible to locate a faint band 3 cm below $tRNA_{4\ minor}^{Lys}$. Both tRNAs were labeled under the same conditions. The major bands and minor bands were the only bands excised from this gel.

The major bands yielded 6×10^6 cpm and 10×10^6 cpm for $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$, respectively. The eluant from band $tRNA_{2\ minor}^{Lys}$ gave 1.4×10^6 cpm while $tRNA_{4\ minor}^{Lys}$ contained 4.3×10^6 cpm.

An autoradiogram of the two-dimensional purification gels for [5'-^{32}P]pCp-labeled $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ is shown in Fig. 2. The lane labeled $tRNA_{5b}^{Lys}$ was part of a split chromatographic peak of $tRNA_5^{Lys}$, and not a separate isoacceptor. The first dimension is displayed 90° to the direction it ran. Both $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ show three distinct bands. The second dimension

Fig. 1. Purification gel for $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ from murine lymphoma. Thirty picomoles each of [5'-^{32}P]pCp-labeled $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ were copurified on a 10% polyacrylamide, 7 M urea gel. Electrophoresis was performed at 350 v until the xylene cyanol FF marker was 25 cm from the origin (about 31 hr, 0° C). The autoradiogram was exposed for 10 sec at room temperature with an intensifying screen.

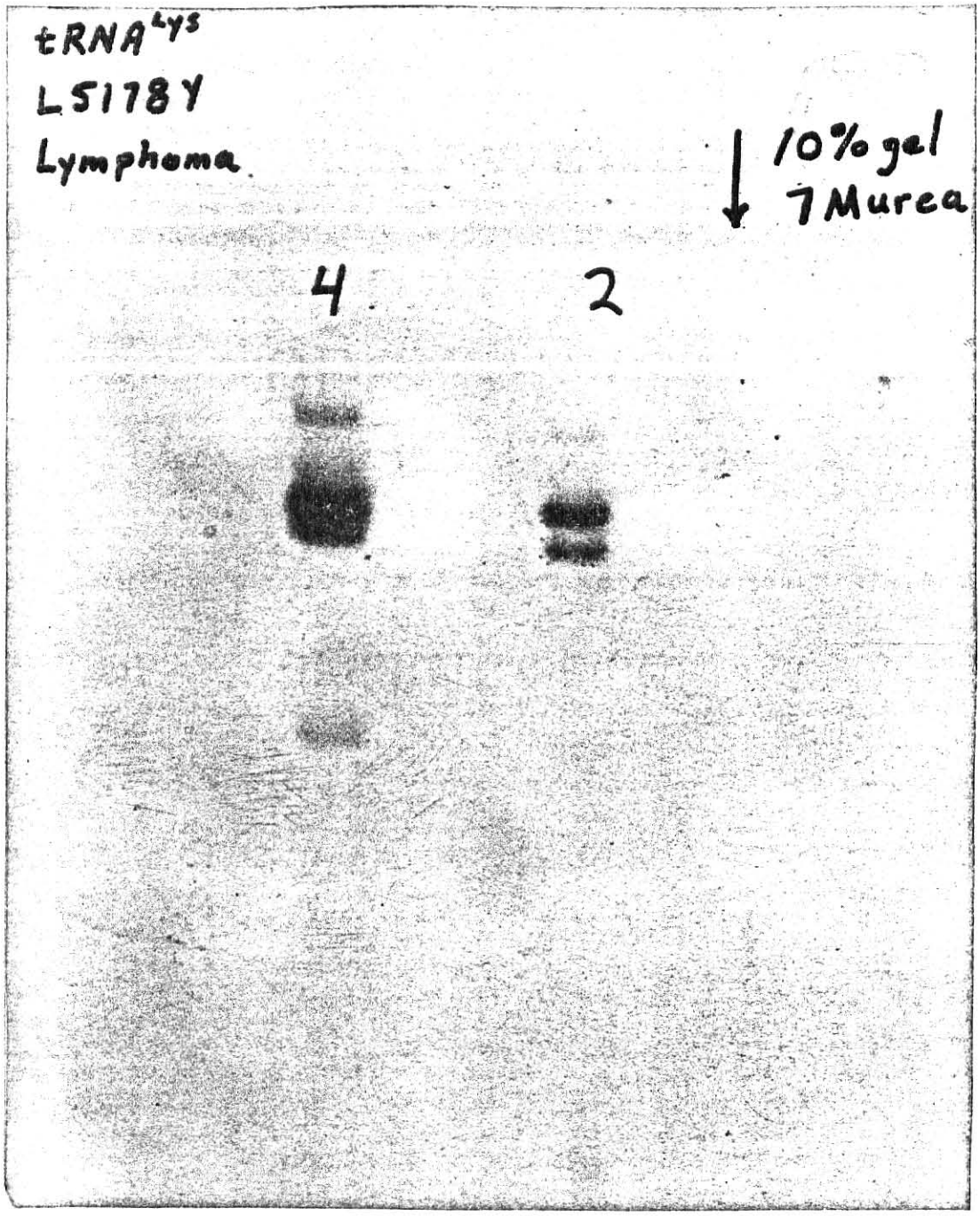

tRNA Lys
L5178Y
Lymphoma
10% gel
7M urea
4
2

Fig. 2. Purification gel for $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$. Thirty picomoles each of [5'-^{32}P]pCp-labeled $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ were copurified on a two-dimensional gel system. The first dimension was a 10% polyacrylamide, 7 M urea gel. Electrophoresis was performed at 350 v until the xylene cyanol FF marker was 16 cm from the origin (about 18.5 hr at 0° C). The autoradiogram was exposed for 20 sec with an intensifying screen. The bands were excised, turned 90°, and placed between the two plates of the second-dimension gel system. The 20% polyacrylamide, 3 M urea second-dimension gel was poured around these gel pieces. Electrophoresis was performed at 350 v for 103 hr at 0° C (xylene cyanol FF marker was run off of the gel). The autoradiogram was exposed for 45 sec at room temperature with an intensifying screen.

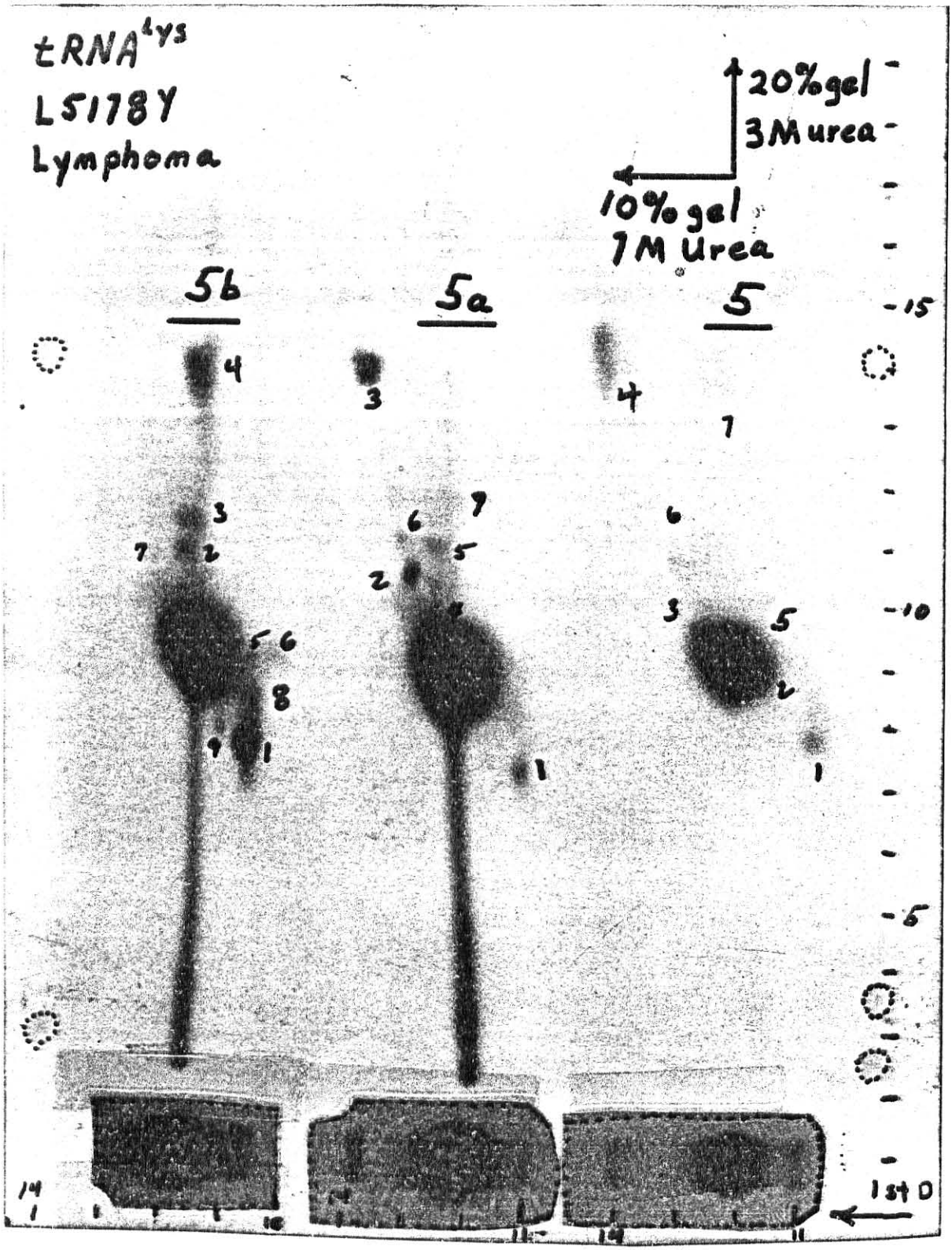

tRNA Lys
L5178Y
Lymphoma
20% gel
3M urea
10% gel
7M urea
5b
5a
5
15
10
5
1st D

Fig. 3. Purification gel for $tRNA_6^{Lys}$. Thirty picomoles of [5'-^{32}P]-pCp-labeled $tRNA_6^{Lys}$ was purified on a 10% polyacrylamide, 7 M urea gel. Electrophoresis was performed at 350 v until the xylene cyanol marker was 27 cm from the origin (about 16.5 hr, at room temperature). The autoradiogram was exposed for 20 sec at room temperature with an intensifying screen.

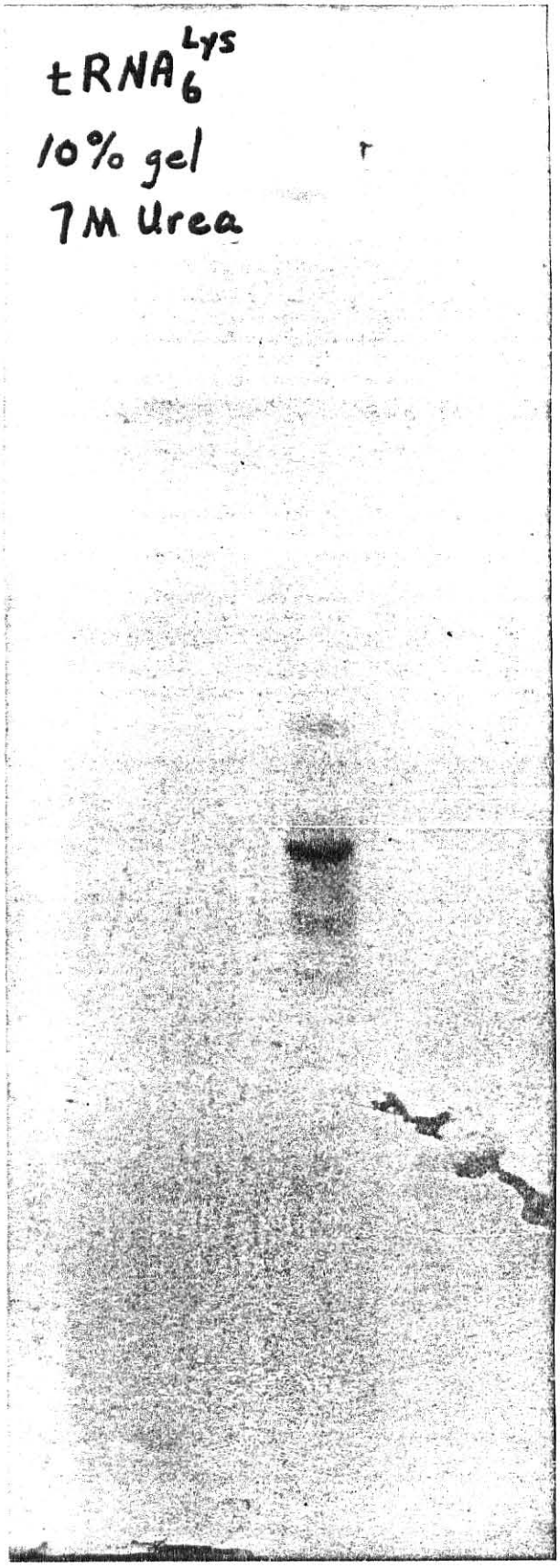
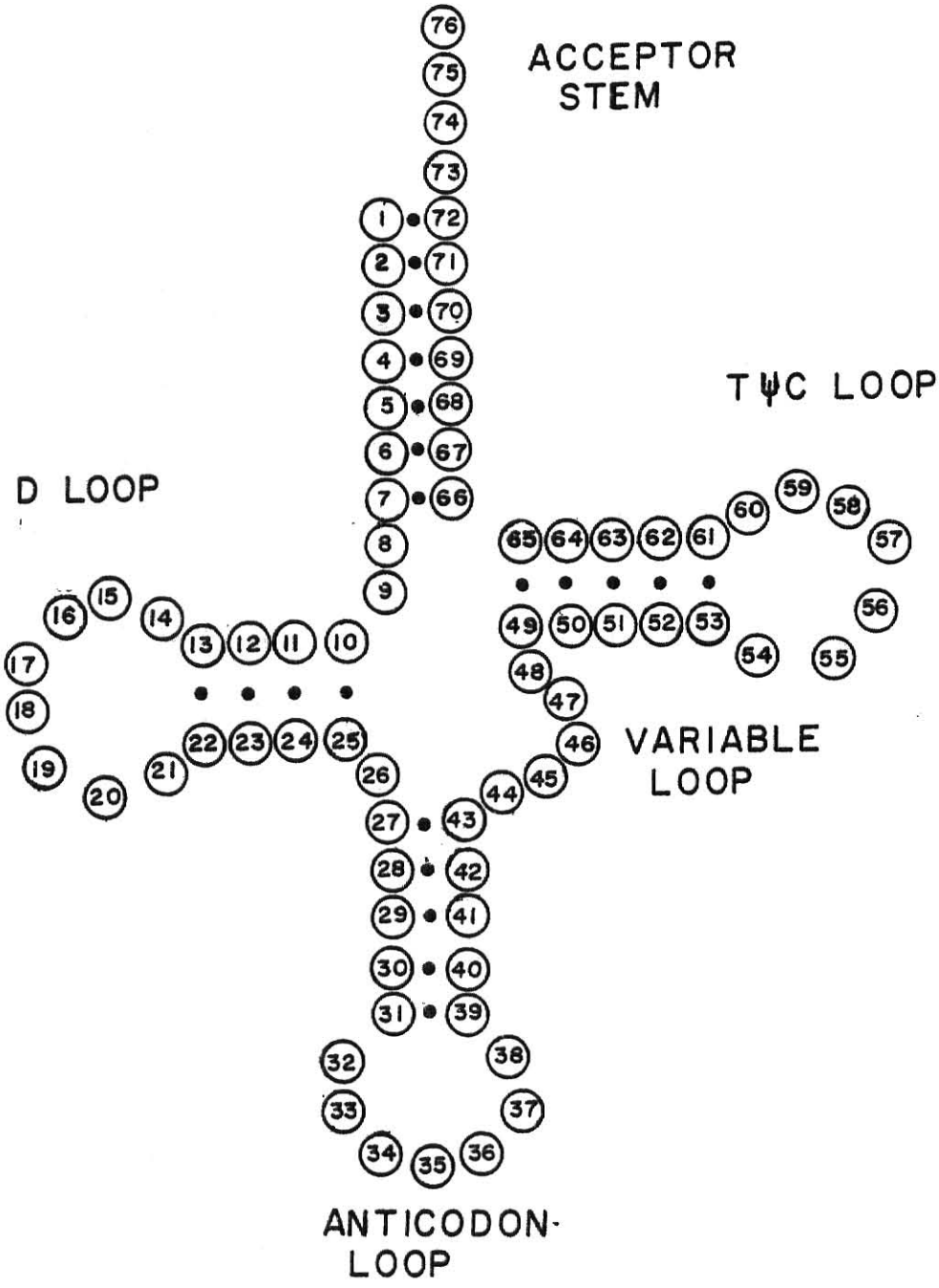

tRNA$_6^{Lys}$
10% gel
7M Urea

Fig. 4. General cloverleaf for lysine tRNA family.


ACCEPTOR
STEM
TψC LOOP
D LOOP
VARIABLE
LOOP
ANTICODON-
LOOP

shows resolution of the components of bands from the first dimension. This produced from 7 to 9 radioactive spots. The major radioactive spots were excised along with spots 5_1, 5_4, $5a_1$, $5a_3$, $5b_1$, and $5b_4$. The counts recovered for $tRNA_5^{Lys}$, $tRNA_{5a}^{Lys}$, and $tRNA_{5b}^{Lys}$ were 1×10^6, 3.7×10^6 and 3.7×10^6 cpm, respectively. The remaining spots were too weak to be accurately sequenced.

The autoradiogram shown in Fig. 3 is of the purification gel for [5'-^{32}P]pCp-labeled $tRNA_6^{Lys}$. It shows a highly labeled, well resolved major band with one faint band 1.8 cm above and two faint bands, 1 cm and 1.8 cm, below the major band. The major band was the only band excised. This purification gel yielded 63,000 cpm of labeled tRNA. This was the second electrophoretic purification of this sample. The tRNA was previously electrophoresed but was stored for a period of time. This protocol was followed because of possible deleterious effects of nucleolytic activity in stored samples.

Unlabeled tRNAs were purified in the same manner as labeled material and detected by a staining procedure (see Experimental Procedures). Single dimension runs were used for all procedures requiring unlabeled material.

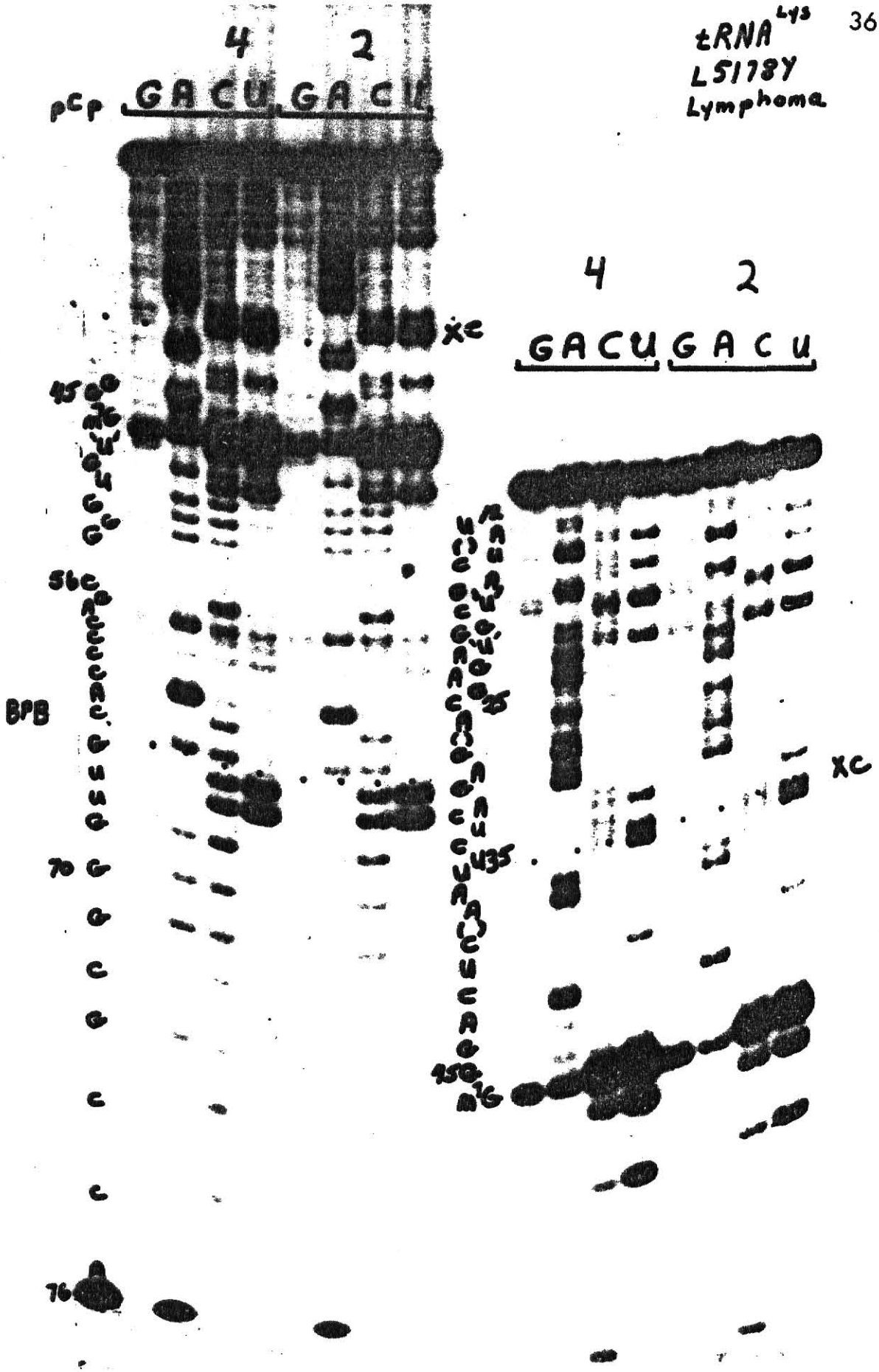
Sequencing of Lysine tRNAs

Fortunately, all of the lysine isoacceptors that were sequenced in our lab contained 76 nucleotides. The basic skeleton for these lysine tRNAs is shown in Fig. 4. This figure provides the numbering system of nucleotides referred to throughout this thesis.

Nucleotide sequences of major and minor species of $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ from murine lymphoma cells. The sequences of the major nucleotides of $tRNA_2^{Lys}$, $tRNA_{2\ minor}^{Lys}$, $tRNA_4^{Lys}$ and $tRNA_{4\ minor}^{Lys}$ were determined by the

Fig. 5. Sequencing ladder from chemical treatment of $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$. Each lane represents a reaction, where the reaction type is signified by the letter symbol of the major base. Each tRNA has eight lanes, four for each addition. Each lane contained 15,000 cpm of labeled tRNA. The second addition was placed on the gel when the xylene cyanol FF marker had traveled 14.5 cm from the origin. With the second addition a sample containing only a dilution of [5'-^{32}P]-pCp was applied to a single lane. This radioactive nucleotide traveled as would the nucleotide representing the 3' terminal adenosine. Each band is labeled to indicate the nucleotide identified. The sequence is read from the bottom up, starting with addition 2 (left hand side). When reading becomes difficult due to lack of resolution addition 1 is read. The overlap can be located by the xylene cyanol FF marker (XC). The autoradiogram was exposed for 7.5 hr at -70° C with an intensifying screen.

tRNA^{Lys}
L5178Y
Lymphoma



chemical sequencing method of Peattie (45). An autoradiogram of the sequencing gel of the major species $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ is shown in Fig. 5. The position of [5'-^{32}P]pCp in the second addition indicates that the last band corresponds to the 3'-terminal adenosine (position number 76). This method permitted sequencing from position 76 through position 12. Addition 2 includes positions 76 through 44 while addition 1 furnished positions 46 through 12.

Positions 58 through 62 were slightly ambiguous probably due to the secondary structure effects resulting from interaction between the run of cytidines with guanosines of the acceptor stem.

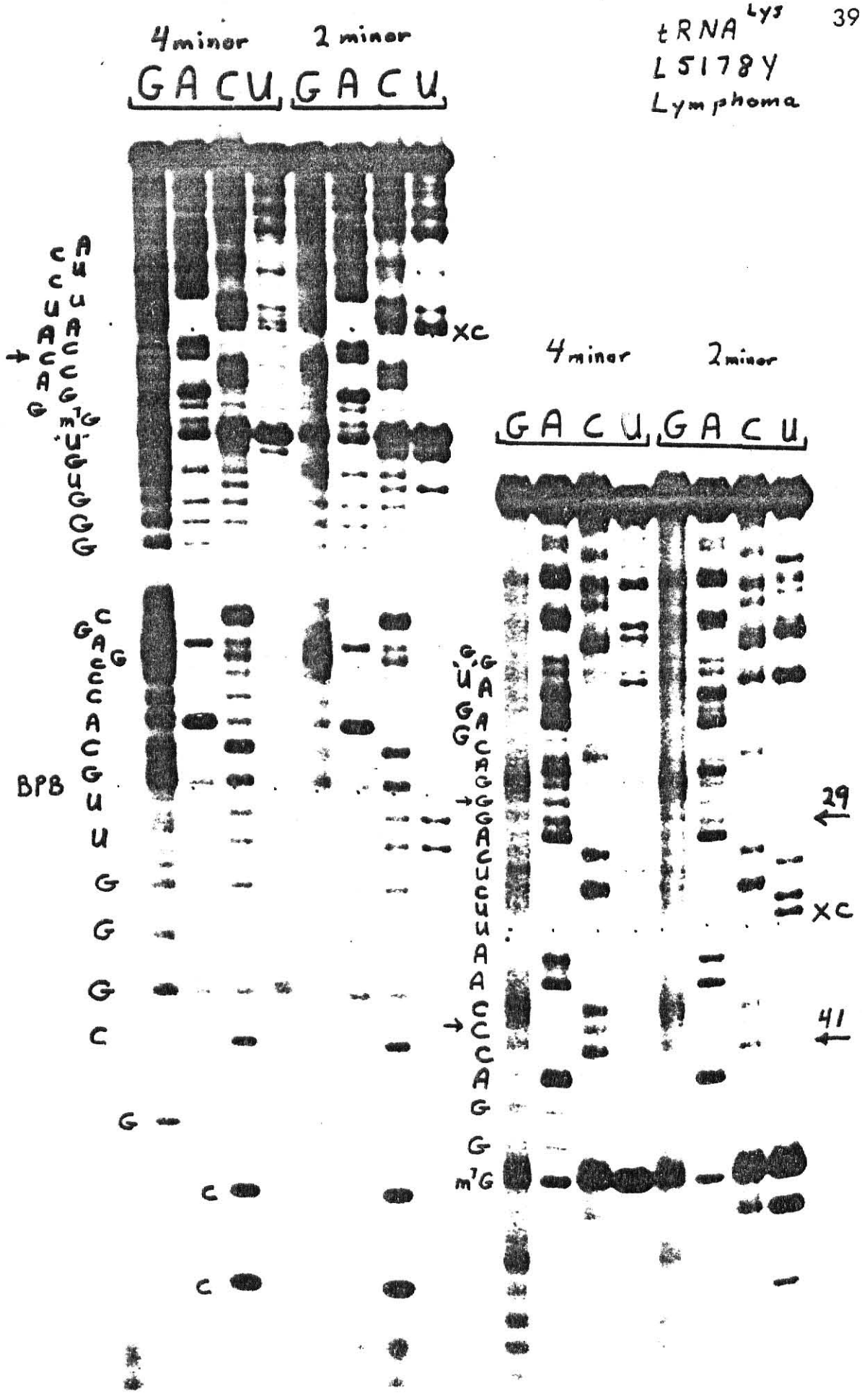
Dihydrouridine nucleotides respond to the chemical treatment like uridine nucleotides. Thus, positions 16, 20, and 47 of $tRNA_2^{Lys}$ and 16 and 47 of $tRNA_4^{Lys}$ are dihydrouridines as will be shown later. The break at m^7G produces a very marked band because it also reacts extensively under the pyrimidine reaction conditions and to a lesser extent under the purine reaction conditions. Gaps between bands generally represent the absence of a break at a modified nucleotide. The positions corresponding to Tm and Ψ are represented by a blank region just above position 56.

The sequence of major nucleotides obtained from the autoradiogram for $tRNA_4^{Lys}$ (Fig. 5) was identical to that of $tRNA_2^{Lys}$ except for ambiguities where dihydrouridines occur. The modified adenosines on the 3' side of the anticodon react as adenosines and are identified later.

The minor species of $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ were also sequenced by the chemical technique, and the results are shown in Fig. 6. These two minor species were sequenced from position 18 through position 75. Both of the sequences were identical to their major counterparts except for a base pair

Fig. 6. Sequencing ladder from chemical treatment of $tRNA_2^{Lys}$ minor and $tRNA_4^{Lys}$ minor. The procedure followed for sequencing of these isoacceptors is the same as that for Fig. 5. Each lane contained 7,000 cpm of labeled tRNA. The autoradiogram was exposed for 15 hr with an intensifying screen.

tRNA^{Lys}
L5178Y
Lymphoma



change in the anticodon stem of each tRNA. At positions 29 and 41 the U:A base pair is substituted by a G:C base pair.

To complete the sequencing of the major species of $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ the method of Stanley and Vassilenko (50) was used with modifications as outlined in Experimental Procedures.

An autoradiograph of a 20% polyacrylamide gel used to separate the nested set of fragments of $tRNA_4^{Lys}$ is shown in Fig. 7. A similar pattern was achieved for $tRNA_2^{Lys}$. The numbers of the bands correspond to the positions in the tRNA of the nucleotides at the 5'-termini of the fragments. The bands resolve at regular intervals with the exception of 61, 59, and 55 in lane 2. Bands 61 and 59 are associated with the high cytidine region in the GTΨC stem. This was also an ambiguous area in the chemical sequencing gel. As explained previously, the anomoly is most likely due to secondary structure interactions. The band represented by 54-55 subsequently was identified as resulting from a fragment containing a 5' Tm dinucleotide. The TmΨ linkage is not hydrolyzed by the formamide treatment; therefore, a fragment ending at the 5' termini with Ψ does not exist.

The 5' terminus of each band was released by treatment with RNase T_2 and identified by thin layer chromatography.

Autoradiograms of chromatograms showing partial results for $tRNA_2^{Lys}$ are shown in Fig. 8 and 9. The sequence is shown starting at the 5' end with nucleotide 1 and continuing through position 7 (Fig. 8). Position 2 indicates two nucleotides present at this position. This may be explained by a phenomenon known as band compression, wherein the tRNA in the preceding band, in the case of the band representing position 1, will appear smaller due to secondary interactions and comigrate with the band just below it.

Fig. 7. Sequencing ladder from formamide hydrolysis of $tRNA_4^{Lys}$. The nested set of [5'-^{32}P]-labeled tRNA fragments produced by formamide hydrolysis (see Experimental Procedures) was resolved on a 20% polyacrylamide, 7 M urea gel to produce this ladder. A second nested set of fragments was resolved on a 12% polyacrylamide, 7 M urea gel resulting in a similar ladder. (The 20% gel was used to increase the resolving power of the system for the smaller oligonucleotides while the 12% gel was used to separate the larger fragments.) Each band is numbered with the nucleotide position identified. The autoradiogram was exposed for 1.5 hr at -70°C with an intensifying screen.

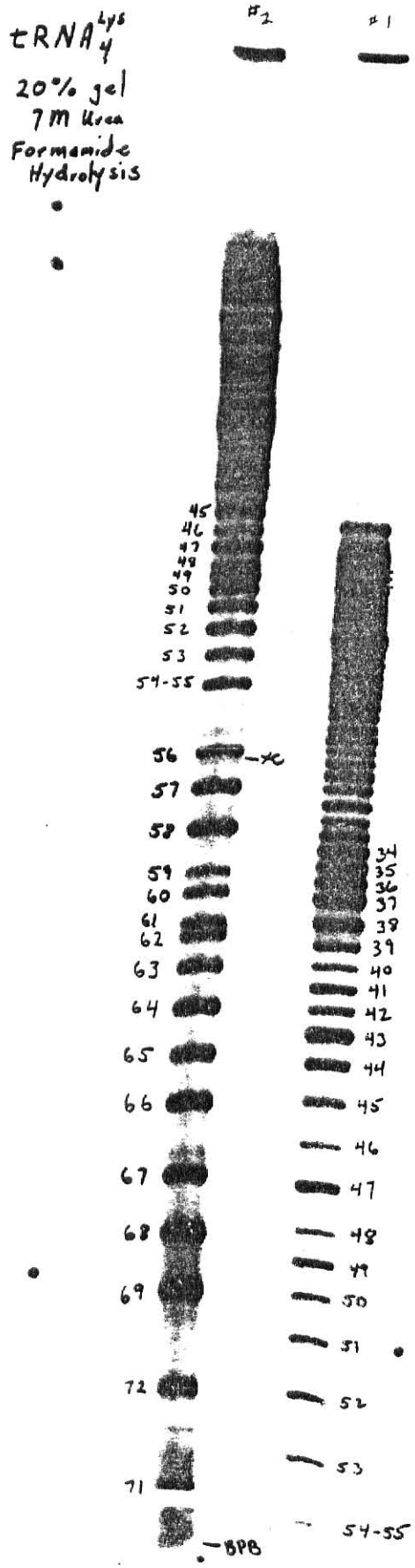
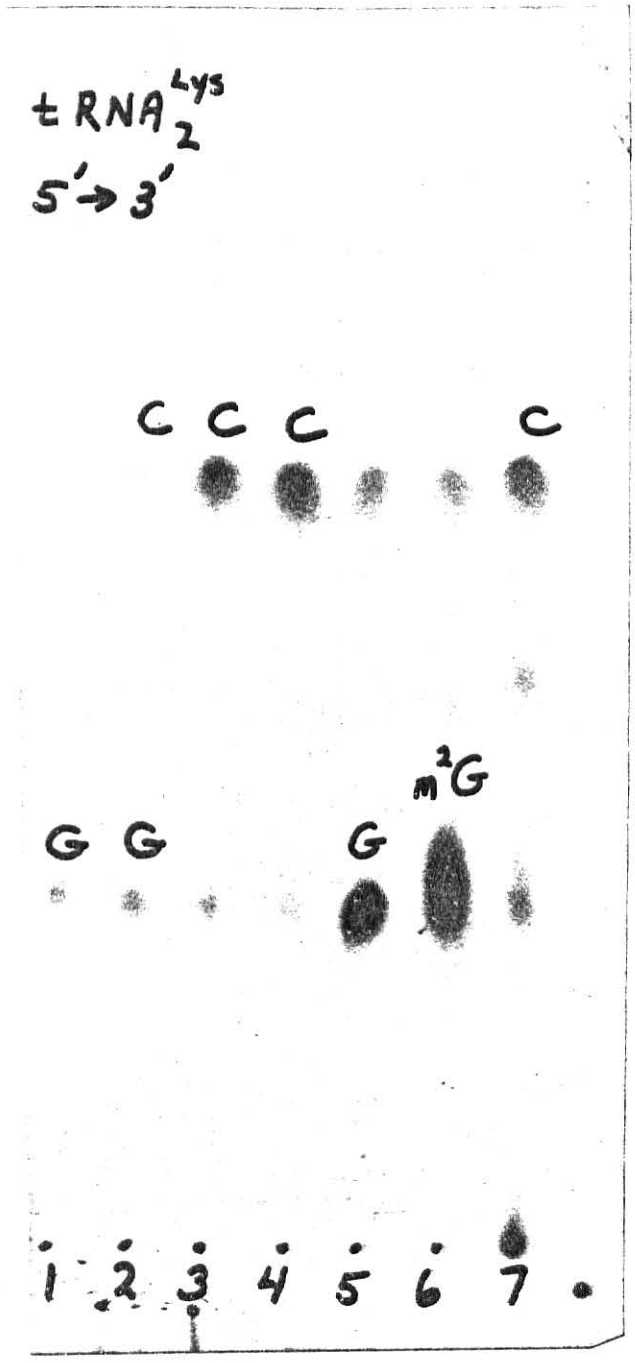

tRNA Lys 4
20% gel
7M Urea
Formamide
Hydrolysis
#2
#1
45
46
47
48
49
50
51
52
53
54-55
56
57
58
59
60
61
62
63
64
65
66
67
68
69
72
71
XC
BPB
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50
51
52
53
54-55

Fig. 8. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_2^{Lys}$ (nucleotides 1-7). Each fragment from the formamide hydrolysis ladder was excised, eluted, and treated with RNase T_2 producing a pNp from the 5'-terminus. Sample from each digest was spotted on one of two PEI-cellulose TLC plates. One plate was chromatographed in 0.55 M ammonium sulfate, and the other was treated with 0.1 M ammonium formate and chromatographed in 1.75 M ammonium formate (see *Experimental Procedures*). The autoradiogram of the PEI-cellulose TLC plate containing nucleotides 1 through 7 is shown. The nucleotides are identified by their relative mobilities as compared to standards located by UV light. The plate shown was chromatographed in ammonium sulfate. The autoradiogram was exposed for 18 hr at -70° C with an intensifying screen. This figure shows the presence of m^2G in position 6.


t RNA$^{Lys}_{2}$
5′→3′
C C C C
G G G
m^{2}G
1 2 3 4 5 6 7

Position 71, which is the complement to position 2, is a guanosine which would hydrogen bond with cytidine, supporting the view that position 2 is indeed a cytidine.

A feature of this sequence not reported by Raba et al. (37) is the m^2G in position 6.

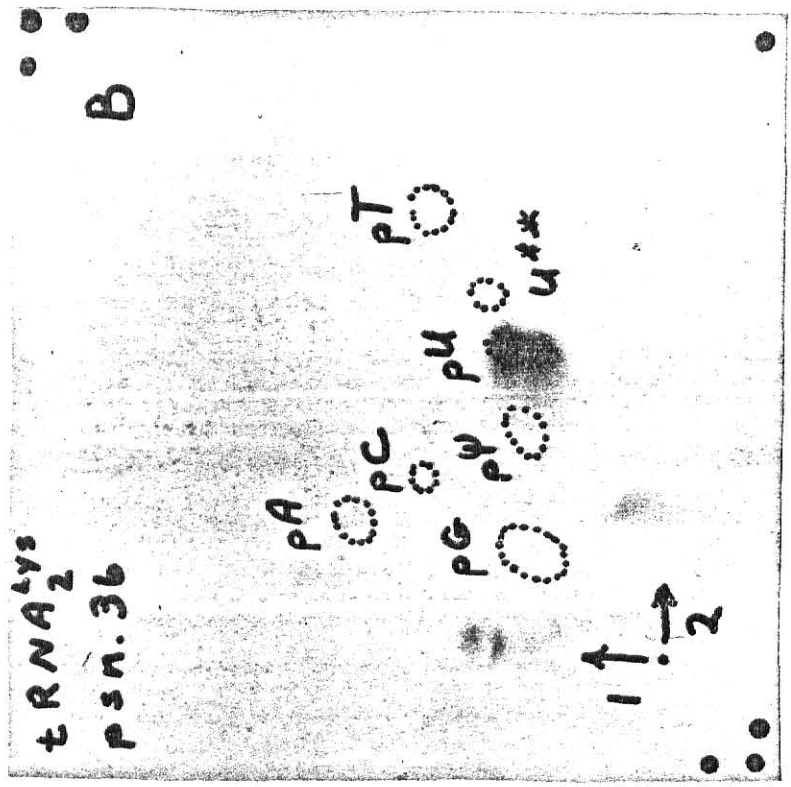
A part of the sequence information from the anticodon loop, stem and variable loop are shown in Fig. 9 (positions 35 through 50). Several minor nucleotides occur in this region including the hypermodified t^6A in position 37. Positions 35 and 36 contain, in addition to pUp, a minor nucleotide that was not identifiable in the chromatographic systems. It runs somewhat like pseudouridine in the one-dimensional systems.

Unfortunately, position 58 of $tRNA_2^{Lys}$ was not determined by this method and remains unidentified. Results of chemical sequencing indicate a minor nucleotide, and I believe that it is m^1A in agreement with the other isoacceptors.

To confirm the types of minor nucleotides in positions 35, 36, 37, and 54-55, these samples were analyzed by two-dimensional chromatography. Results of two-dimensional chromatography are shown in Fig. 10. Results from the nucleotide in position 35 were identical to these of position 36. Although the minor nucleotide occurring along with pU appears to be pΨ in one system, this assignment is not supported in the second system, but identification also is not possible.

Confirmation of the identification of t^6A was attempted in a two-dimensional system (Fig. 11). A spot corresponding to pt^6A was present, but the mobility of the major spot was not that of pt^6A. Further work indicated that the concentration of nuclease P_1 used was not sufficient to convert pt^6Ap to pt^6A, and that position 37 is t^6A.

Fig. 9. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_2^{Lys}$ (nucleotides 35-50). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. The autoradiogram shows the mobilities of U**, t^6A, Ψ, m^7G, D, and m^5C.


B
tRNA$_2^{Lys}$
psn. 36
pA
pC
pG
pΨ
pU
U**
pT
1
2

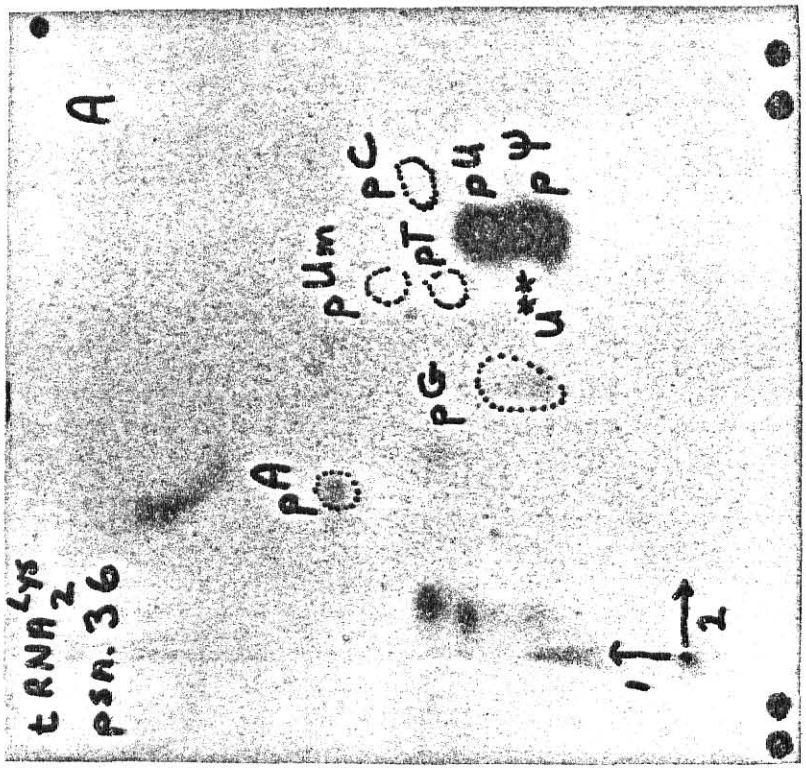

A
tRNA$_2^{Lys}$
psn. 36
pA
pUm
pC
pT
pG
pU
pΨ
U**
1
2

Fig. 10. Two-dimensional chromatogram for identification of position 36 from $tRNA_2^{Lys}$. The solution remaining after the two one-dimensional TLCs was treated with nuclease P_1, producing pNs from pNps, and chromatographed two-dimensionally on a 10 cm x 10 cm cellulose TLC plate in either solvent system 1 or solvent system 2 (see Experimental Procedures). Fig. 10A is the autoradiogram of the cellulose TLC plate for position 36 chromatographed in solvent system 1. The TLC plate shown in Fig. 10B was chromatographed in solvent system 2. The nucleotide was identified as U and some U** from their relative mobilities as compared to standards located by UV light.

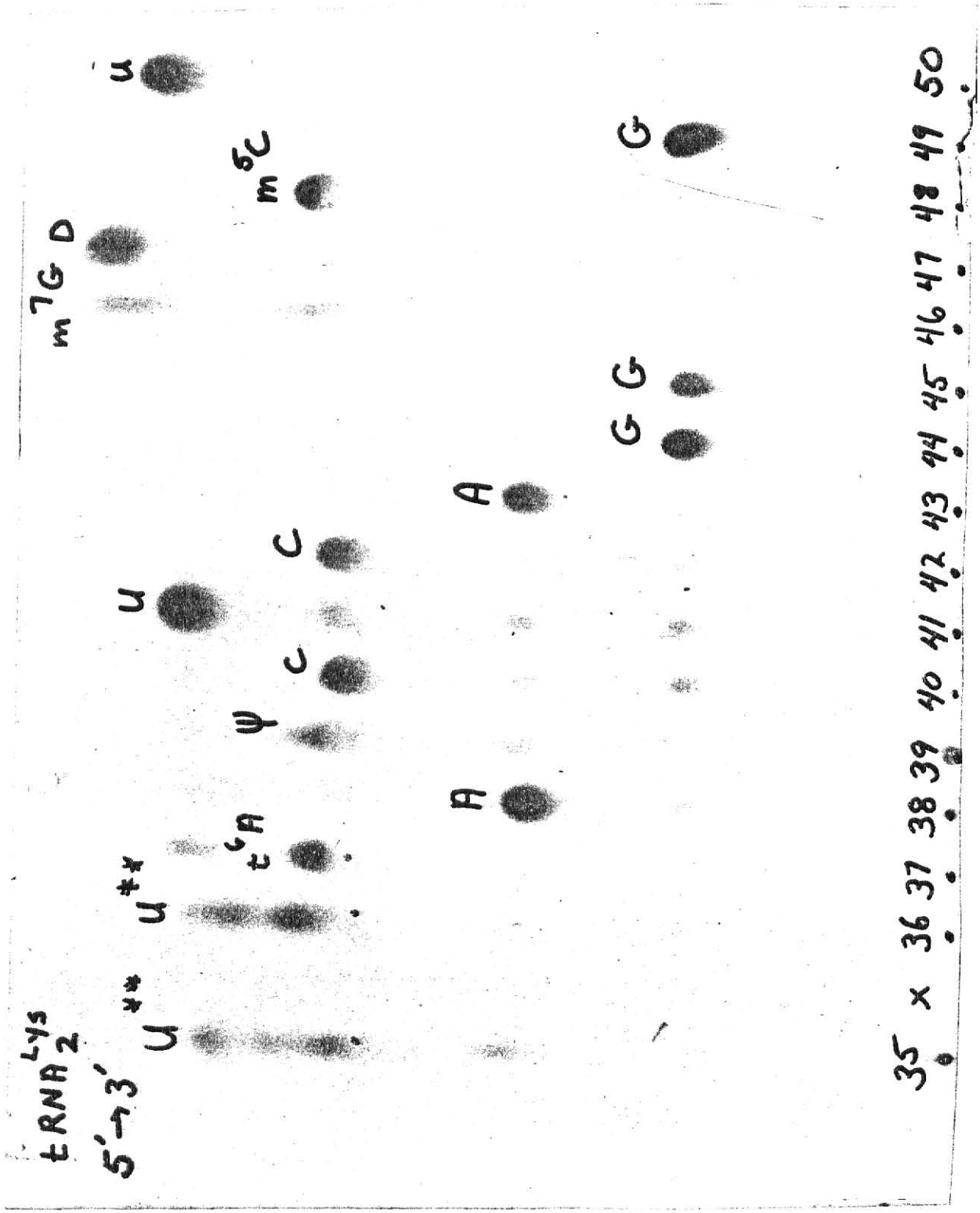
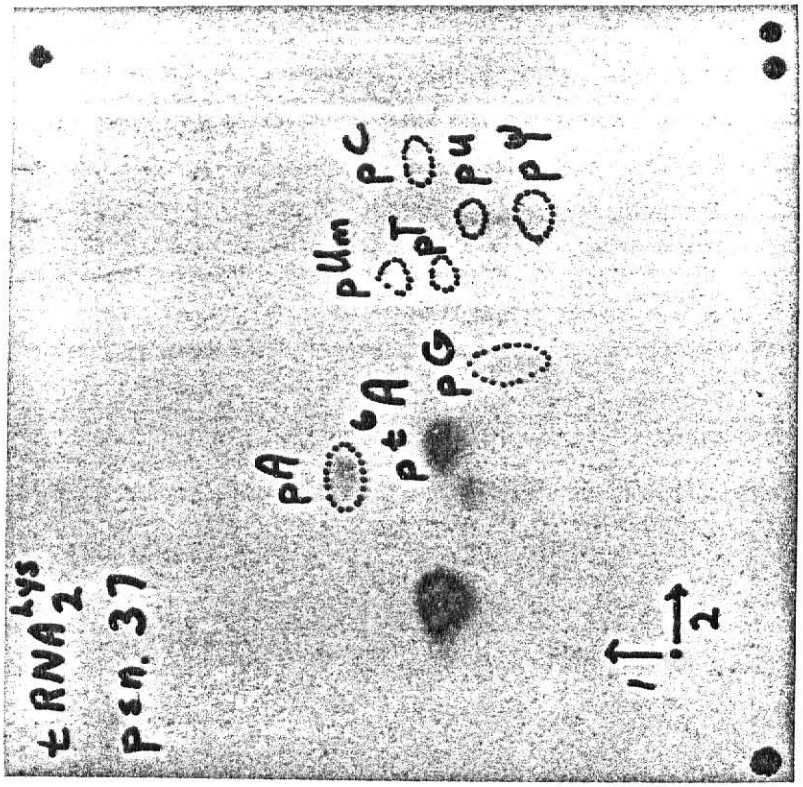

tRNA$_2^{Lys}$
5'→3'
U** U** t^{6}A ψ C U C A A G G m^{7}G D m^{5}C U
35 × 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50

Fig. 11. Two-dimensional chromatogram for identification of position 37 from $tRNA_2^{Lys}$. The procedure followed for producing this TLC plate was the same as that for Fig. 10. The nucleotide identified here is t^6A.


tRNA Lys 2
Psn. 37
pA
pt6A
pG
pUm
pT
pC
pU
pY
1
2

Chromatographic results for position 54-55 gave no useful information. It appears that this is a dinucleotide with considerable resistance to cleavage by nuclease P_1.

Autoradiograms of chromatograms from a one-dimensional system providing partial sequence information for $tRNA_4^{Lys}$ are shown in Fig. 12 to 15. Nucleotides 1-16 are shown in Fig. 12 with the exception of position 2. The band with this nucleotide initially was not excised from the gel, but subsequently was recovered and yielded a cytidine nucleotide.

Again, an m_2G is present in position 6 as well as in position 10 where it is expected on the basis of a published sequence (37). The band for position 10 also contained the band for position 11, which gave a cytidine nucleotide. This occurrence of two nucleotides in one lane is due to the phenomenon of band compression during electrophoresis.

Only one minor nucleotide, pΨp, occurs in the partial sequence shown in Fig. 13 (nucleotides 17-31).

Results covering the anticodon region are presented in Fig. 14. Lanes 34, 35, and 36 are the positions comprising the anticodon, CUU. As noted in the sequence of $tRNA_2^{Lys}$, an unknown minor nucleotide also occurs in the 3' side of the anticodon along with the uridine nucleotide expected. The nucleotide was not identified.

There is a small amount of t^6A in position 37 of the autoradiogram. The major radioactive product at this position is an unidentified minor nucleotide migrating above pUp.

The relative mobility of the dinucleotide pTmΨp is shown in Fig. 15.

The total nucleoside composition was determined by the postlabeling method of Randerath et al. (69). Autoradiograms of the cellulose sheets

Fig. 12. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_4^{Lys}$ (nucleotides 1-16). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. This readout shows the presence of m^2G in positions 6 and 10. Positions 10 and 11 are found in the same lane due to band compression during electrophoresis.

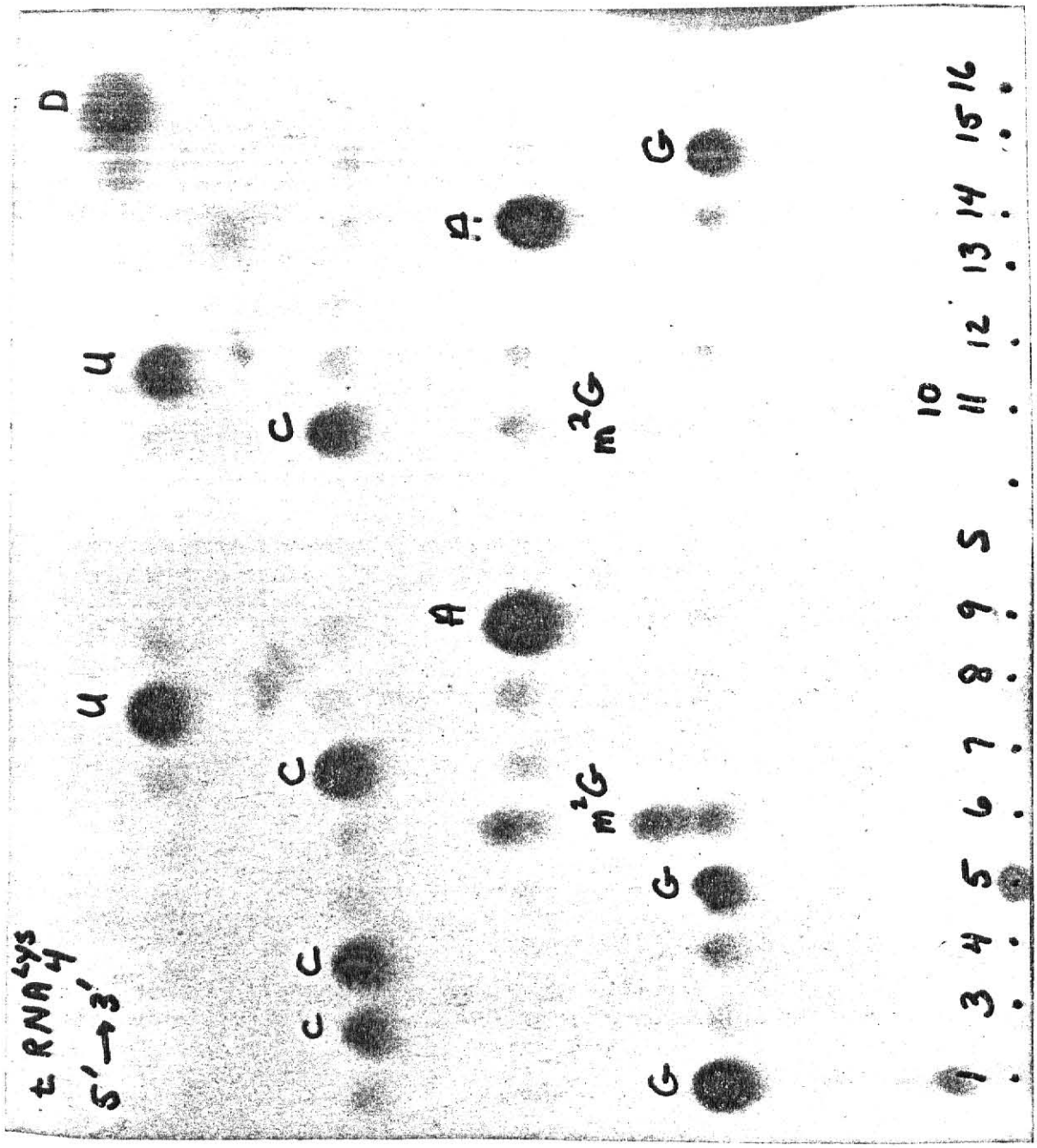

t RNA$^{Lys}_4$
5' → 3'
D
U
U
C
C
C
C
A
A
m^{2}G
m^{2}G
G
G
G
1 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Fig. 13. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_4^{Lys}$ (nucleotides 17-31). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. The relative mobility of pseudouridine is shown in lane 27.

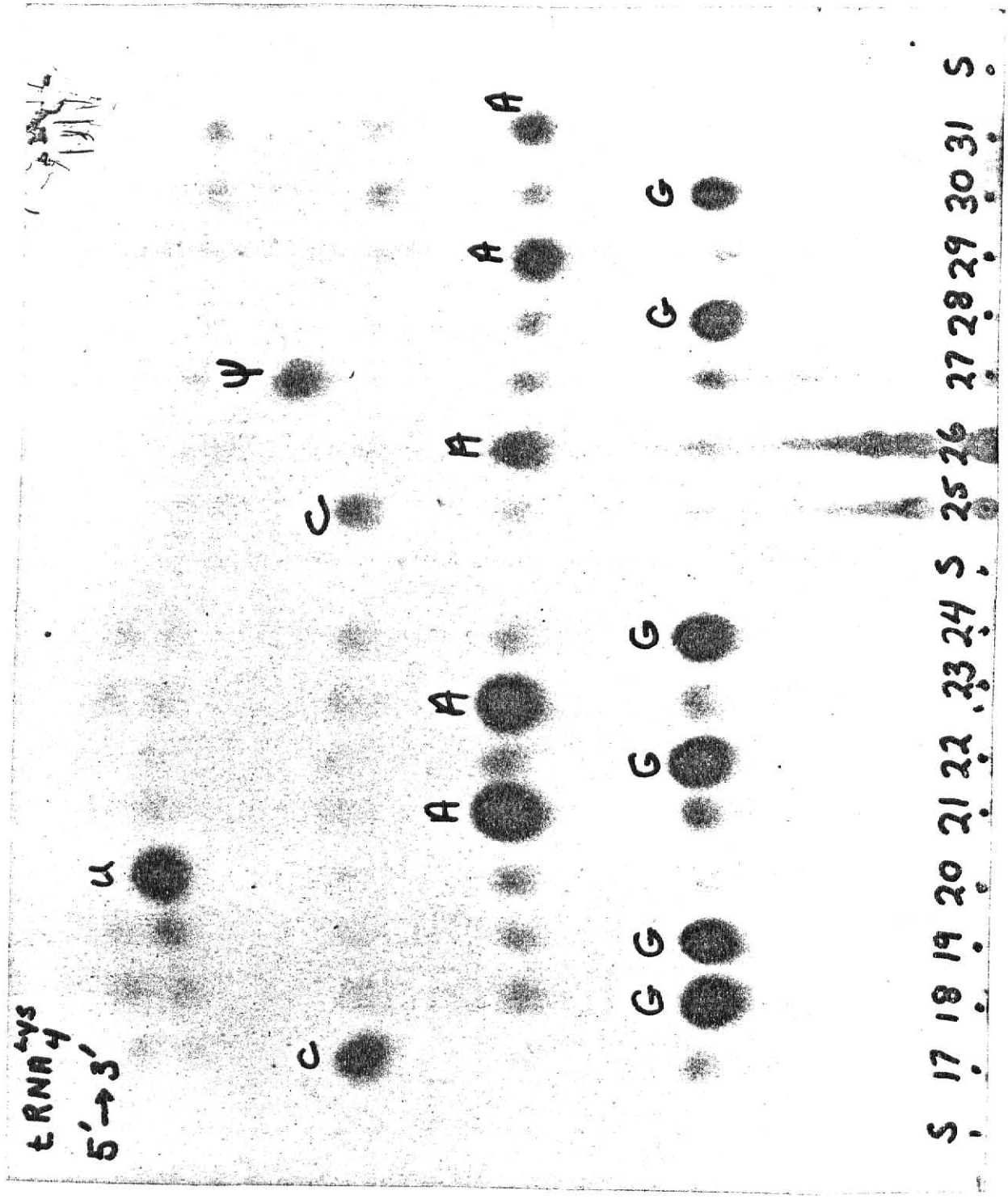

tRNA^{Lys}
5'→3'
U
C
Ψ
C
A A
A A
G G
G G
G G
A A
S 17 18 19 20 21 22 23 24 S 25 26 27 28 29 30 31 S

Fig. 14. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_4^{Lys}$ (nucleotides 30-44). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. The relative mobilities for U, U**, "t^6A", t^6A, and pseudouridine are shown in this readout.

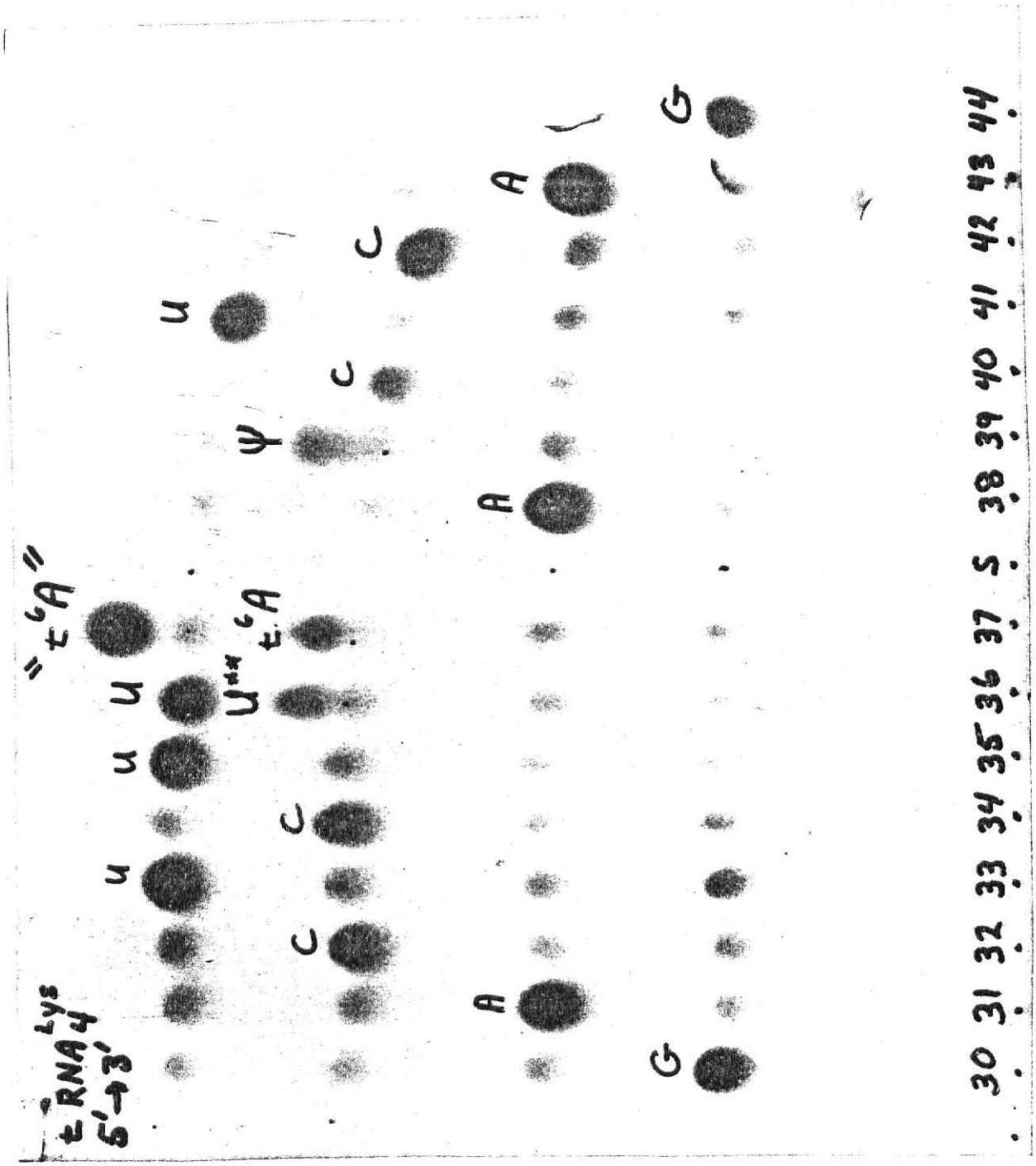

"t⁶A"
t⁶A
30 31 32 33 34 35 36 37 S 38 39 40 41 42 43 44

Fig. 15. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_4^{Lys}$ (nucleotides 51-56). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. The dinucleotide TmΨ, is shown in lane 54-55.

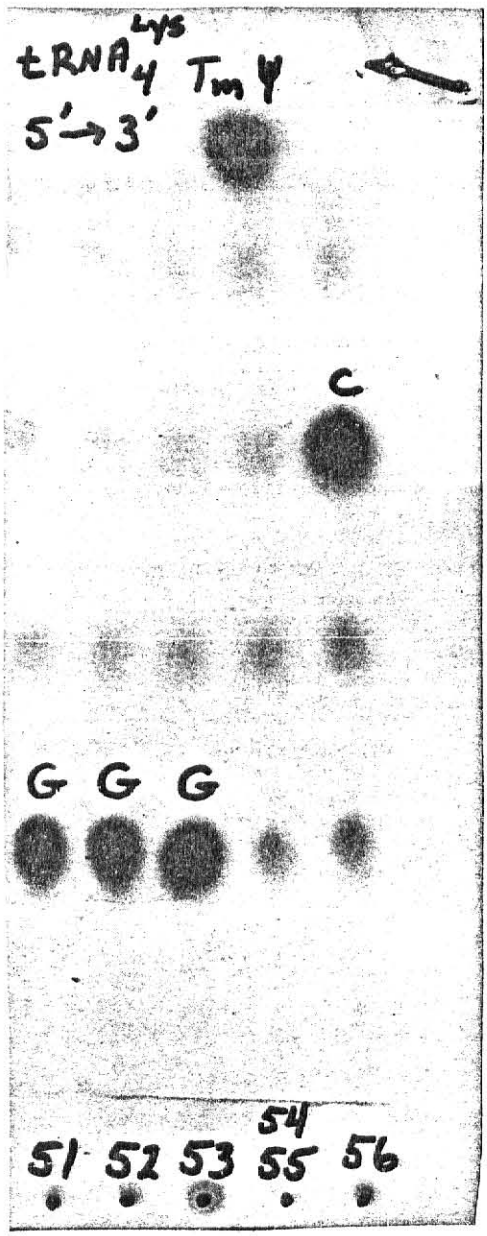

tRNA4 Lys Tm Ψ
5'→3'
C
G G G
51 52 53 54 55 56

Fig. 16. Total nucleoside composition of $tRNA_2^{Lys}$. The procedure followed was that of Randerath et al. (69)(see Experimental Procedures). The total nucleoside composition confirms the type and quantities of nucleosides found from the "sequence readouts" for $tRNA_2^{Lys}$.

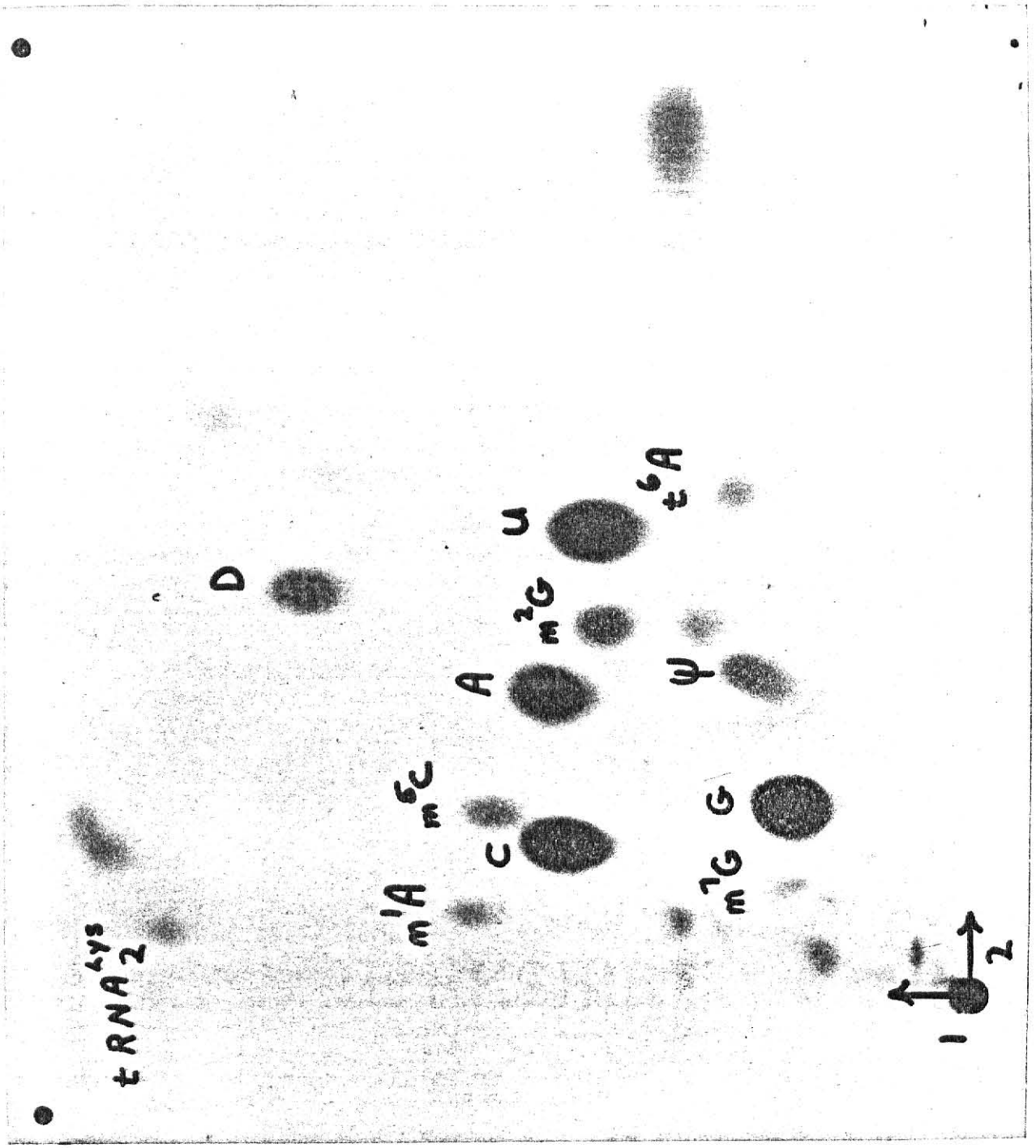

tRNA$^{Lys}_2$
D
m^{1}A
m^{5}C
C
A
m^{2}G
U
t^{6}A
ψ
m^{7}G
G
1
2

Fig. 17. Total nucleoside composition of $tRNA_4^{Lys}$. The procedure followed was that of Randerath et al. (69)(see Experimental Procedures). The total nucleoside composition of $tRNA_4^{Lys}$ is identical to the total nucleoside composition of $tRNA_2^{Lys}$.

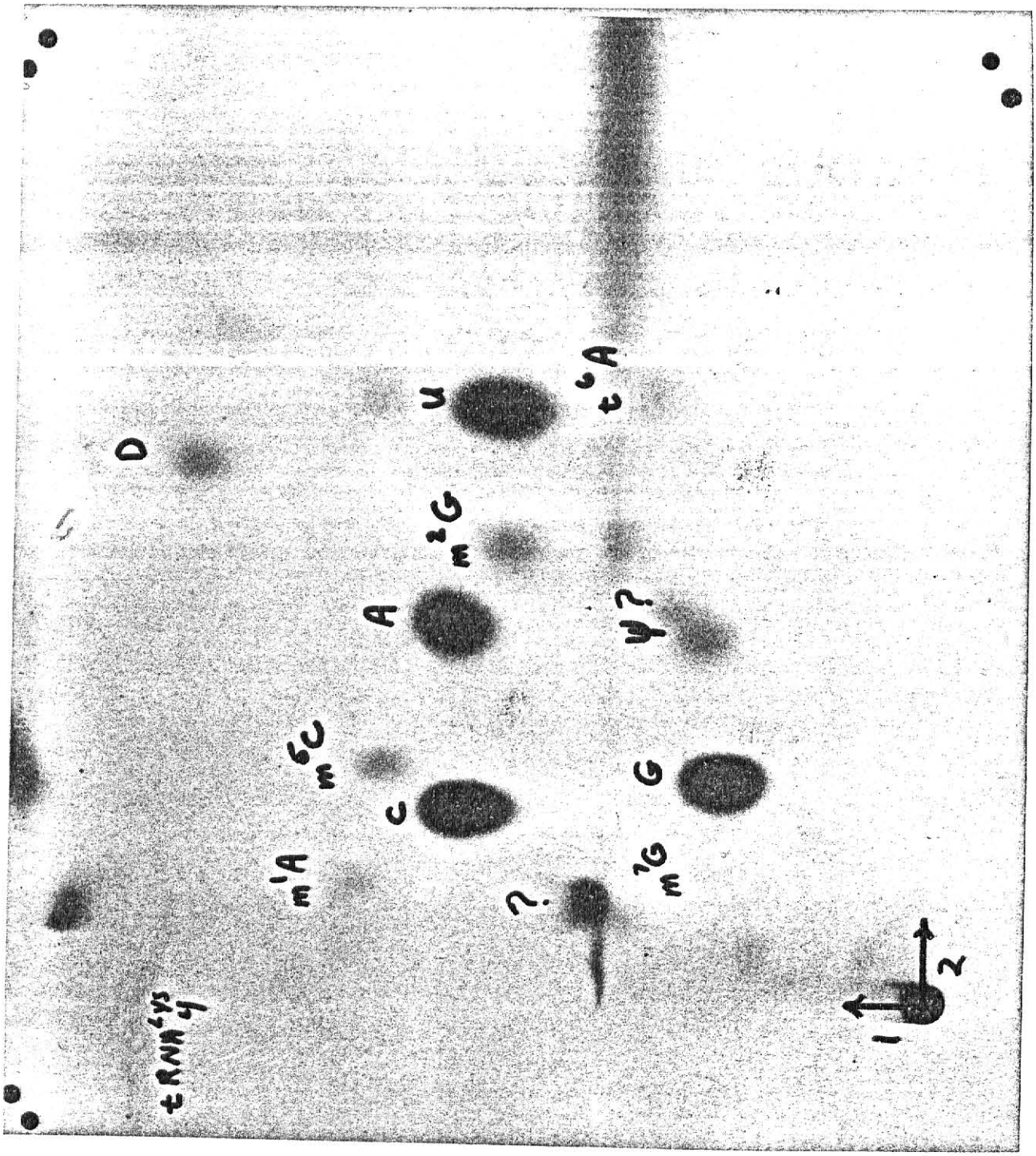

tRNA$^{Lys}_{4}$
m^{1}A
m^{5}C
C
?
m^{1}G
G
A
m^{2}G
Ψ?
D
U
t^{6}A
1
2

TABLE 1

SUMMARY OF SEQUENCING RESULTS FOR $TRNA_2^{LYS}$

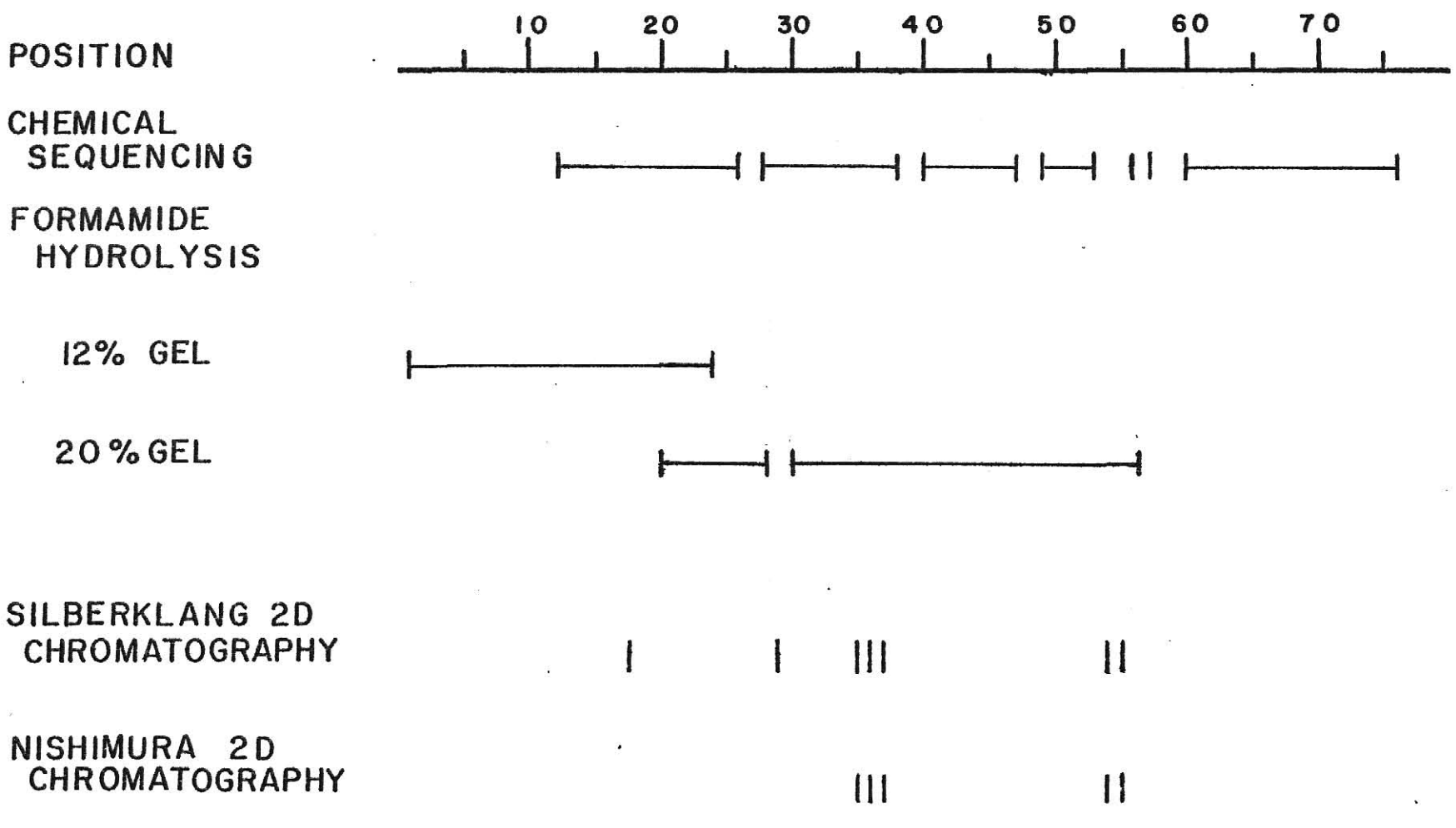


TABLE 2

SUMMARY OF SEQUENCING RESULTS FOR $TRNA_4^{LYS}$

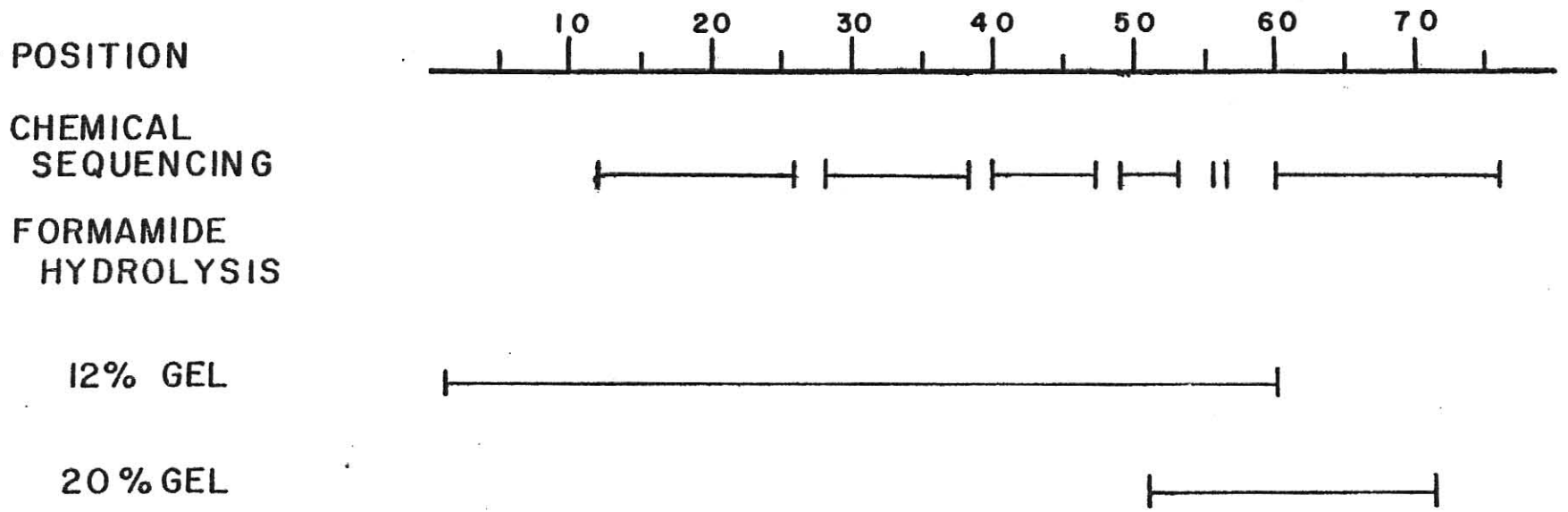


Fig. 18. Nucleotide sequence for $tRNA_2^{Lys}$.

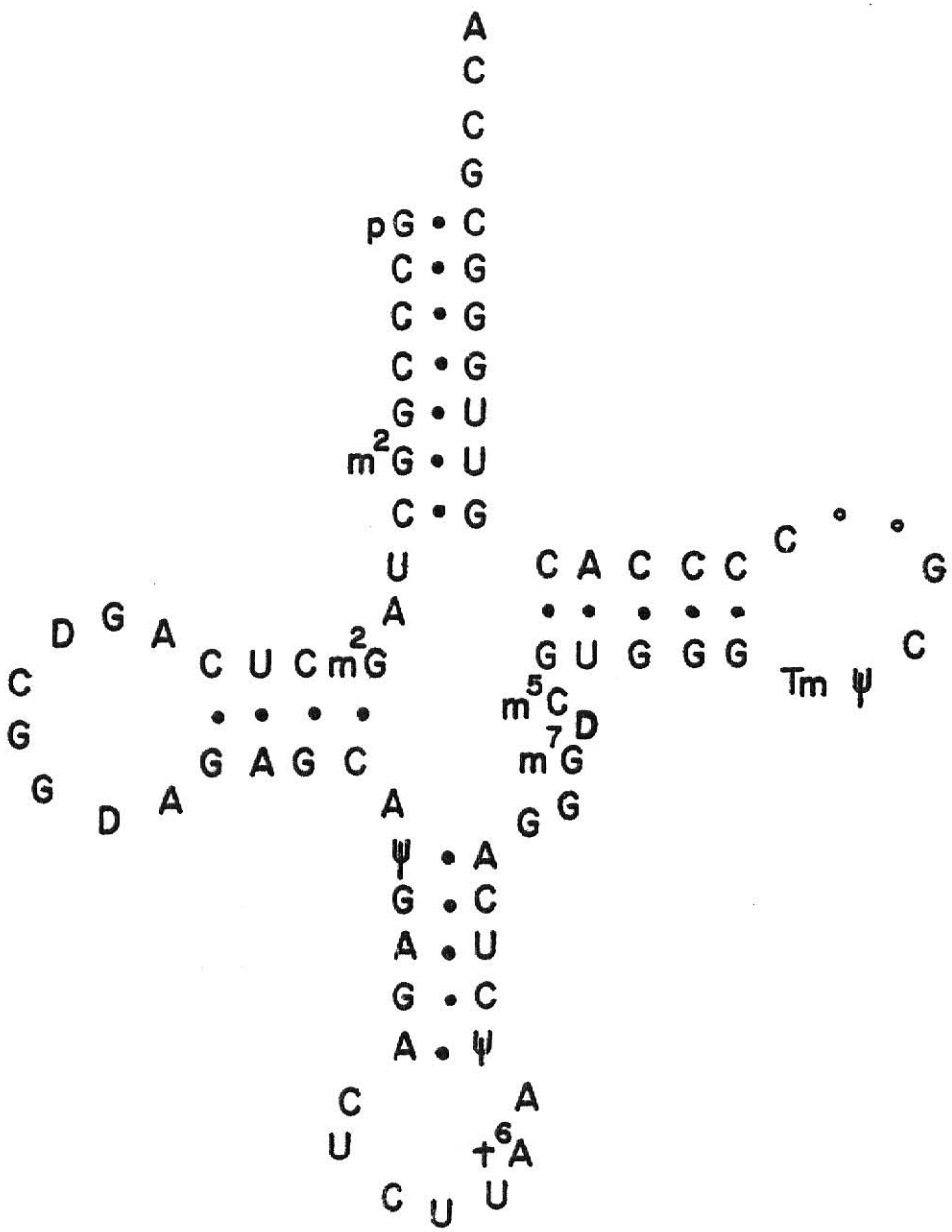
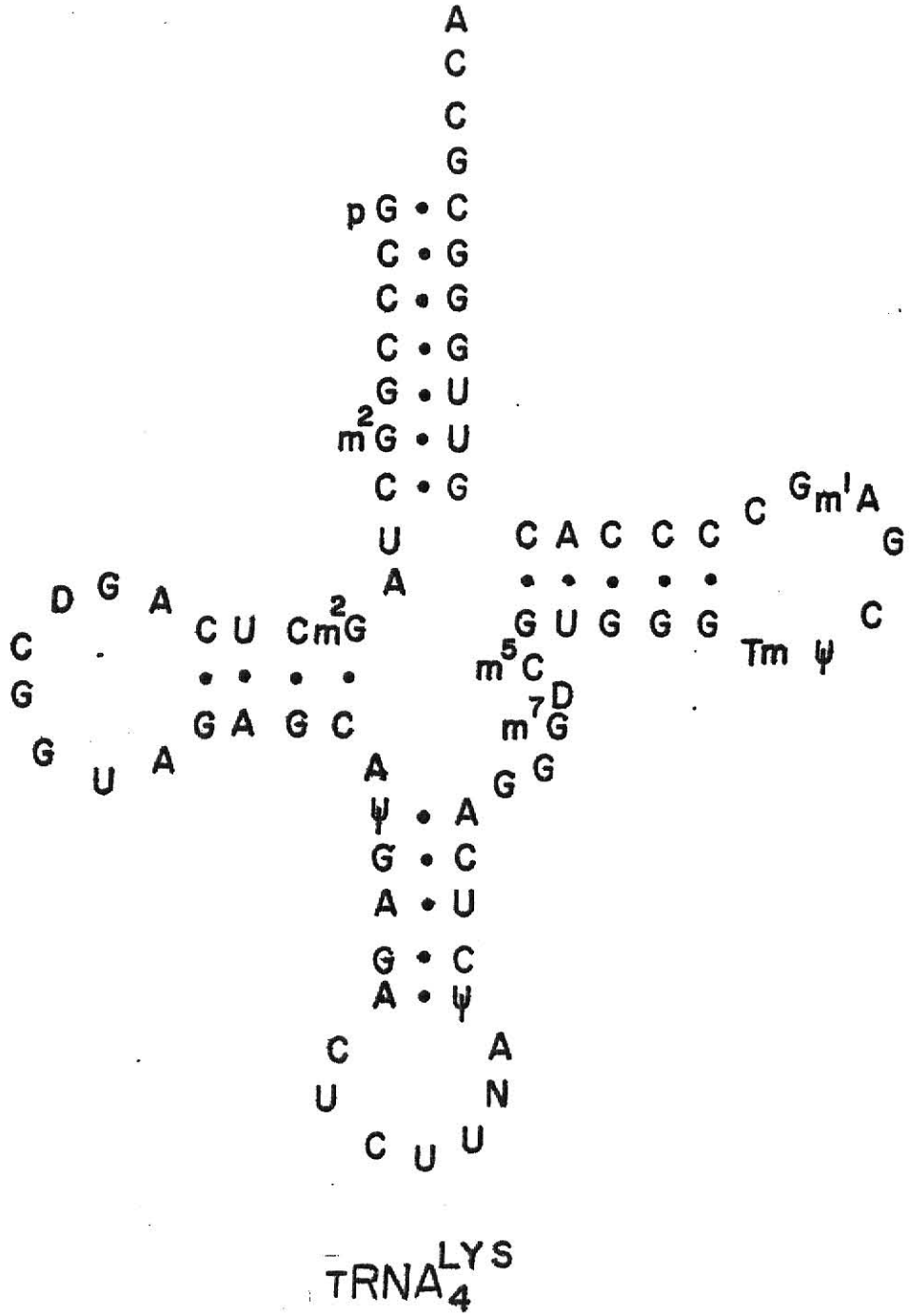

A
C
C
G
pG • C
C • G
C • G
C • G
G • U
m2G • U
C • G
U
A
D G A
C U C m2G
G A G C
D A
C A C C C
G U G G G
Tm ψ
m5C
m7G
D
G G
ψ • A
G • C
A • U
G • C
A • ψ
C A
U +6A
C U U
TRNA2 LYS

Fig. 19. Nucleotide sequence for $tRNA_4^{Lys}$.


A
C
C
G
pG • C
C • G
C • G
C • G
G • U
m2G • U
C • G
U
A
D G A
C U Cm2G
G A G C
G U A
A
ψ • A
G • C
A • U
G • C
A • ψ
C A
U N
C U U
C A C C C
G U G G G
C G m1A G
C
Tm ψ
m5C
m7D
m7G
G G
TRNA4LYS

are shown in Fig. 16 and 17 for $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$, respectively. Both tRNAs contain the same minor nucleotides.

The various methods used to determine the nucleotide sequences of $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ are summarized in Tables 1 and 2, respectively.

The derived sequences are shown in Fig. 18 and 19.

Sequences of $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ from murine lymphoma cells. The positions of major nucleotides were determined by the chemical sequencing method of Peattie (45). An autoradiogram of the sequencing gel for $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ is shown in Fig. 20. The lanes that are labeled $tRNA_{5b}^{Lys}$ are actually a second set of $tRNA_5^{Lys}$. These lanes will not be discussed. The sequence produced starts with the 3' terminal adenosine, position 76, proceeds through to the dihydrouridine loop, terminating with position 11. Positions 12, 27, 34, 39, 48, 49, and 54-59 were not identified within these sequences due to modifications or ambiguities. The reactions displayed very similar patterns as found in the sequencing gel in Fig. 5, i.e. the distinct m^7G bands with dihydrouridine giving a band in the uridine lane.

The sequences of major nucleotides presented for $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ are identical to the sequence reported by Raba et al. (37) for $tRNA_5^{Lys}$ from rabbit liver.

Positions of minor nucleotides were determined by the procedure using formamide hydrolysis to generate a sequencing ladder. Partial sequences are shown in Fig. 21 and 22 for $tRNA_5^{Lys}$ and Fig. 23 and 24 for $tRNA_{5a}^{Lys}$.

In Fig. 21 and 23, m^2G appears in position 6 and 10 for both isoacceptors as it did for $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$.

The anticodon region for $tRNA_{5a}^{Lys}$ is shown in Fig. 24. The minor

Fig. 20. Sequencing ladder from chemical treatment of $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$. The procedure followed for sequencing of these isoacceptors is the same as that for Fig. 5. Each lane contained 5,000 cpm of labeled tRNA. The autoradiogram was exposed for 21 hr at -70° C with an intensifying screen.

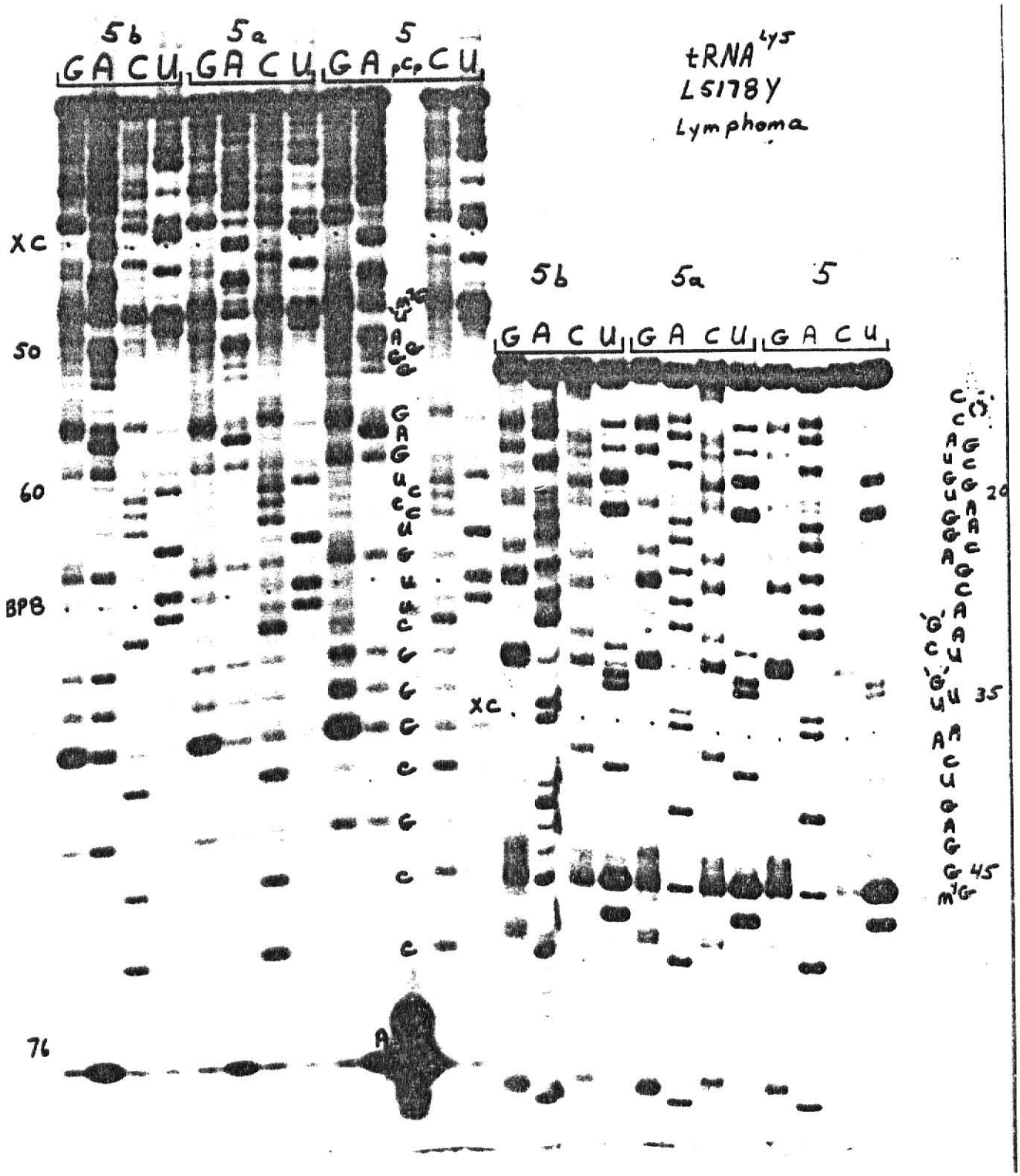

5b
5a
5
G A C U G A C U G A pC, C U
tRNA Lys
L5178Y
Lymphoma
XC
50
60
BPB
76
5b
5a
5
G A C U G A C U G A C U
XC
20
35
45

Fig. 21. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_5^{Lys}$ (nucleotides 6-22). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. This partial readout shows m^2G in positions 6 and 10.

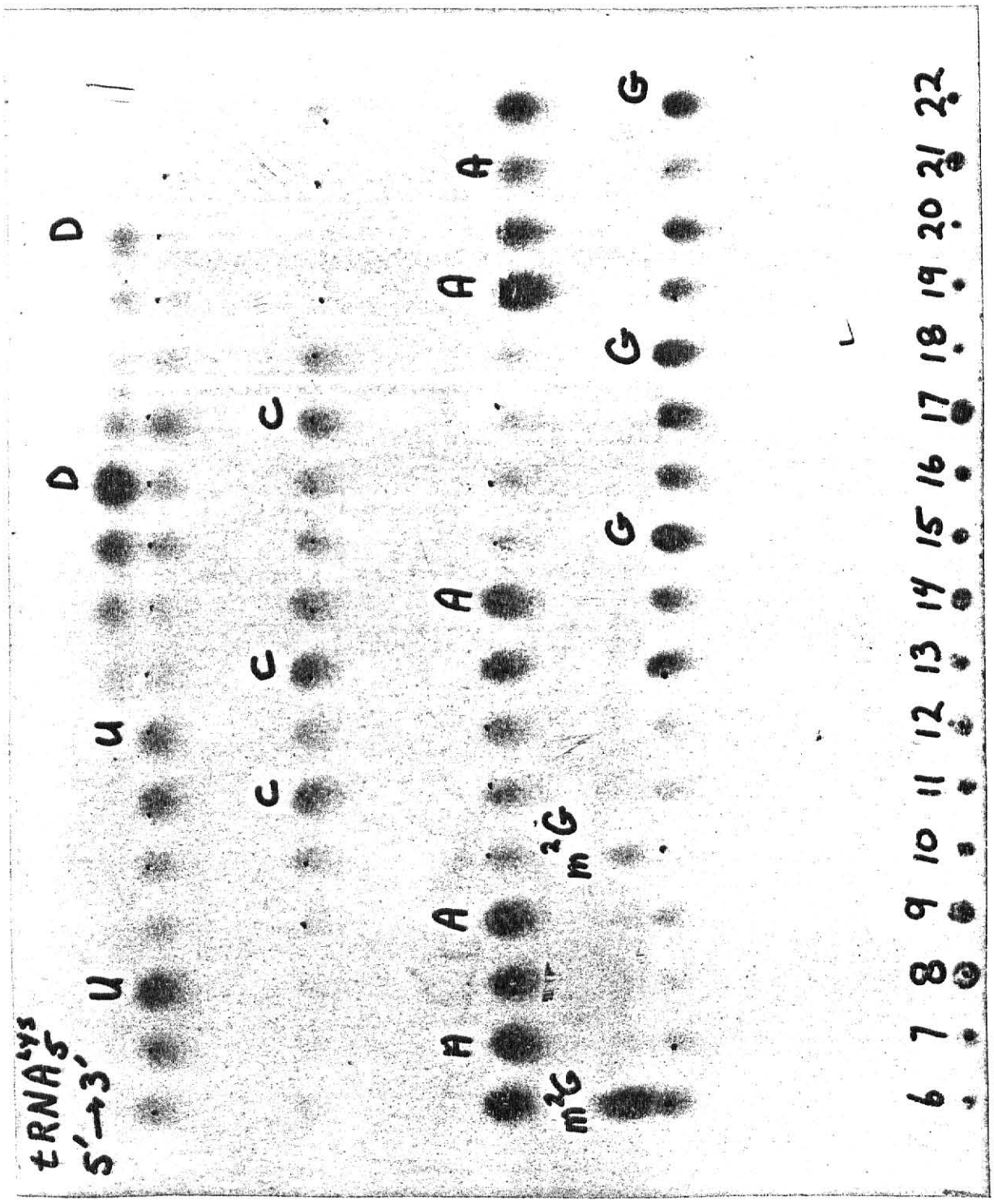

tRNA^{Lys}
5'→3'
U
U
C
C
D
D
C
C
D
A
A
A
A
m^{2}G
m^{2}G
G
G
G
6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22

Fig. 22. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_5^{Lys}$ (nucleotides 36-52). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. Position 37, of $tRNA_5^{Lys}$, is occupied by the hypermodified adenosine, ms^2t^6A. This is the only difference between $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$.

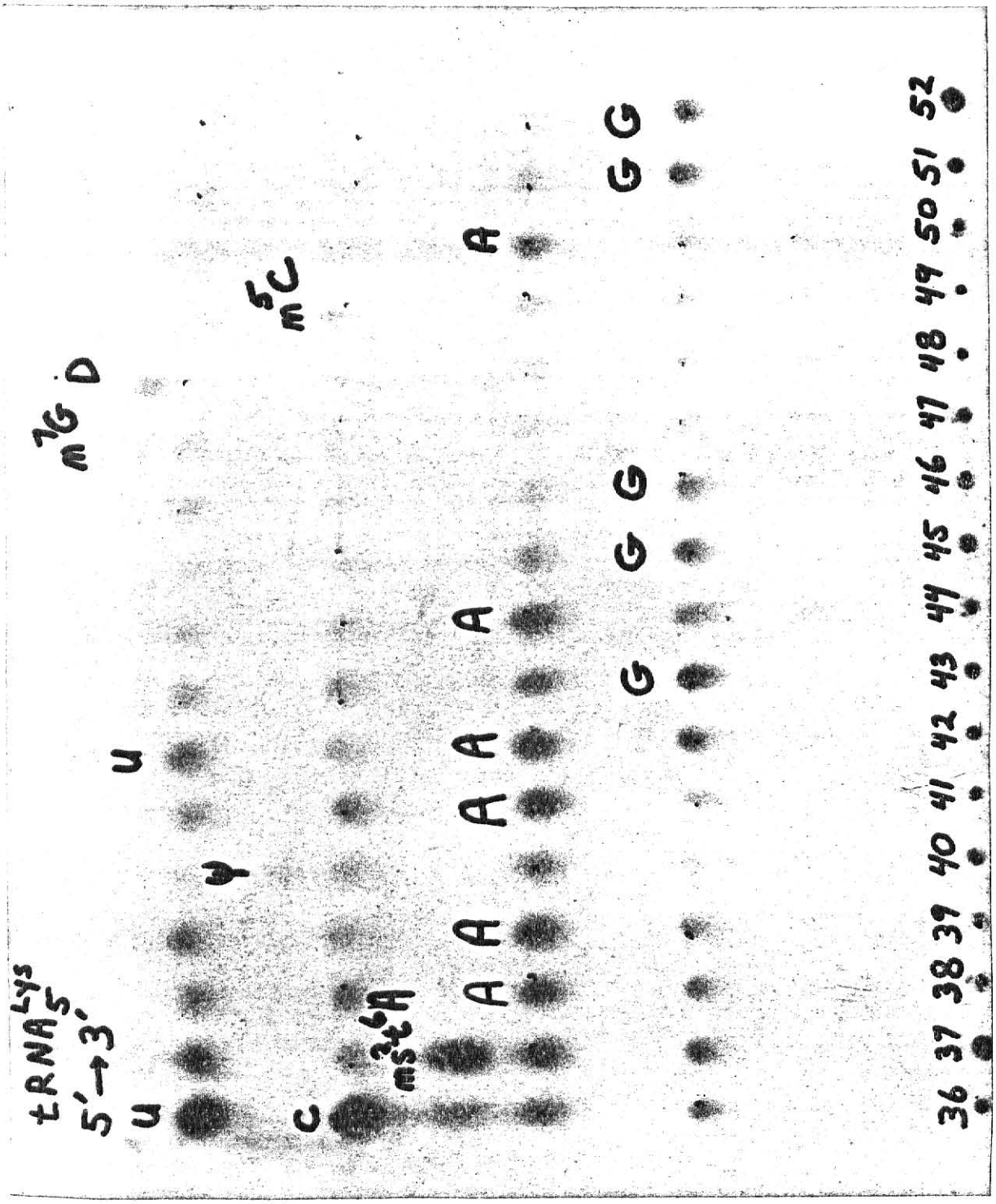

tRNA$^{Lys}_{S}$
5'→3'
U
C
$ms^{2}{}^{6}A$
U
Ψ
$m^{1}G$
D
$m^{5}C$
A A A A A
A
G G G G G
36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52

Fig. 23. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_{5a}^{Lys}$ (nucleotides 4-19). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. This readout shows the m^2G present in positions 6 and 10 in $tRNA_{5a}^{Lys}$. This feature is also shown in Fig. 21.

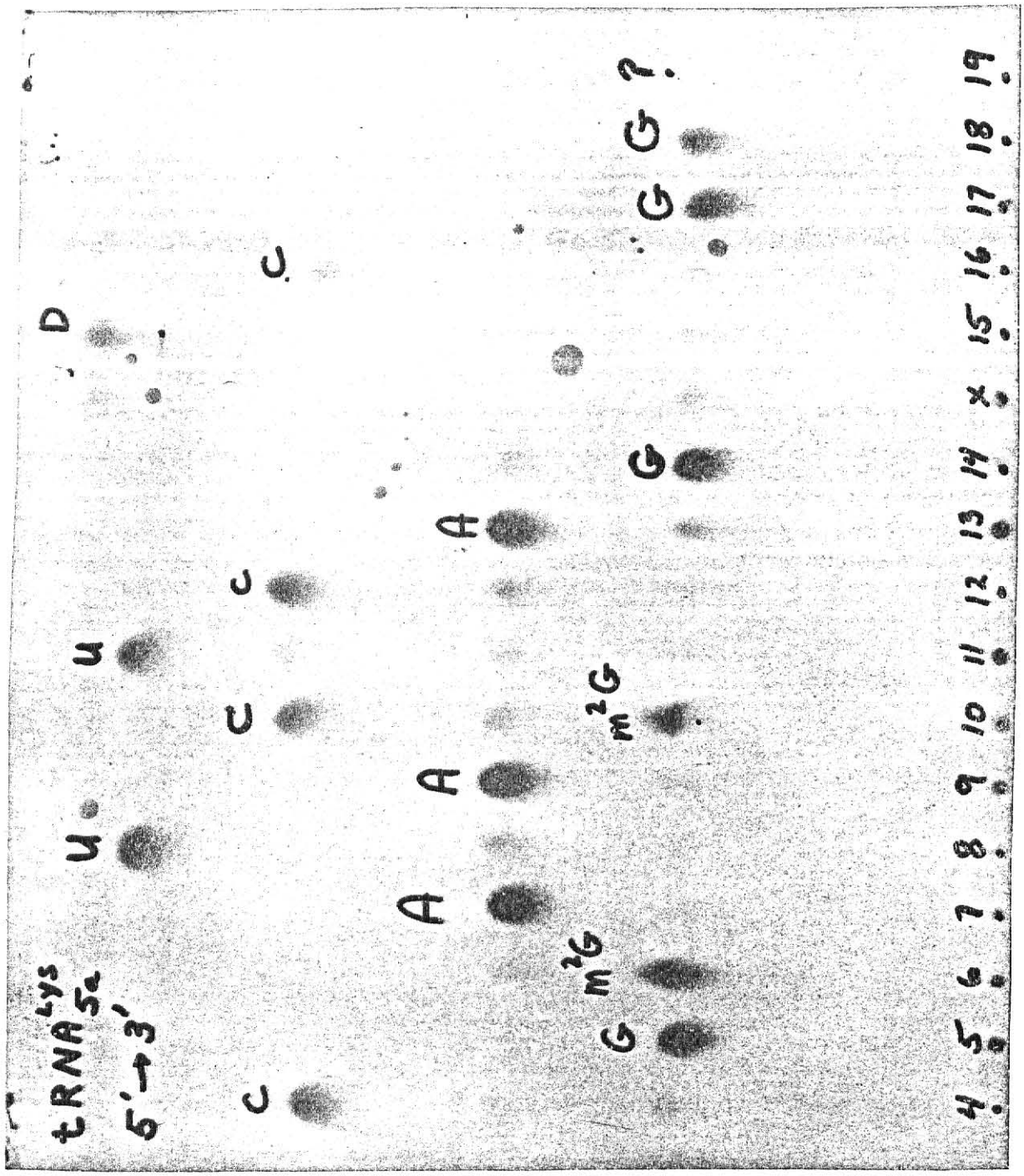
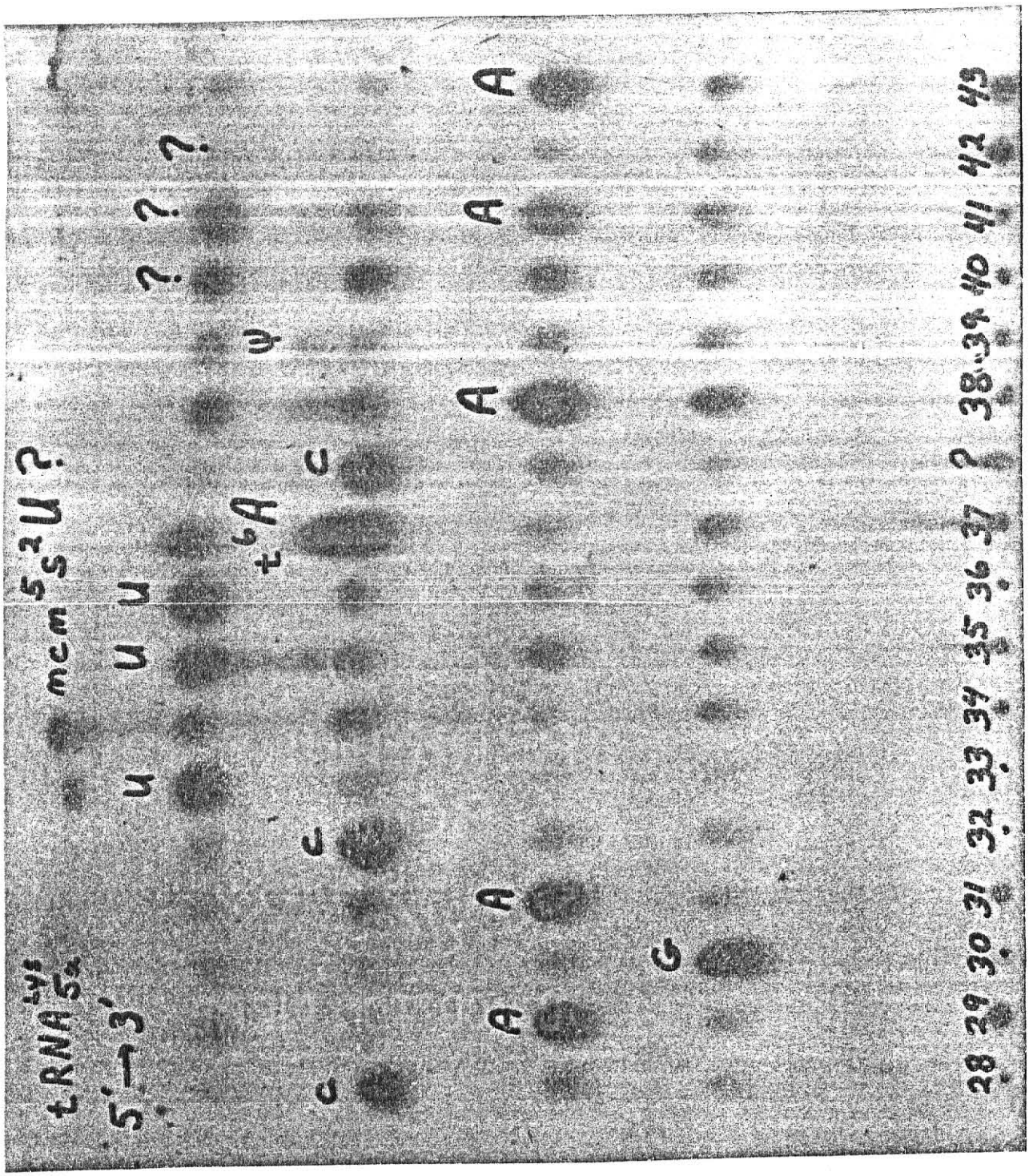

tRNA Lys Sa
5'→3'
C
U
U
C
C
A
A
A
G
m2G
G
m2G
C
U
C
A
G
G
G ?
D
4 5 6 7 8 9 10 11 12 13 14 x 15 16 17 18 19

Fig. 24. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_{5a}^{Lys}$ (nucleotides 28-43). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. The focal point of this partial sequence is the occurrence of mcm^5s^2U in position 34 and t^6A in position 37. Position 34 in $tRNA_5^{Lys}$ also contained mcm^5s^2U.


tRNA Lys Sa
5' — 3'
mcm5s2U ?
U U U
? ? ?
ψ
+6A
c c
A A
A A
c
A
G
28 29 30 31 32 33 34 35 36 37 ? 38 39 40 41 42 43

nucleotide in lane 34 is identified as mcm^5s^2U with some reservations, which are discussed later.

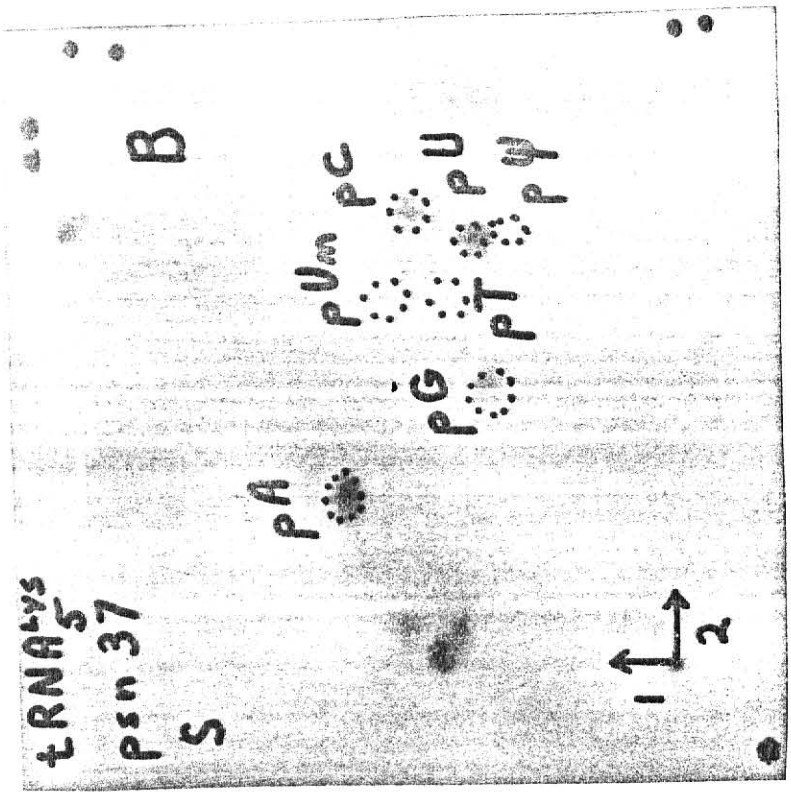
An interesting difference occurs in the base adjacent to the 3' side of the anticodon (position 37). In $tRNA_5^{Lys}$ this is the minor base ms^2t^6A. However, in $tRNA_{5a}^{Lys}$ this position contains the less modified t^6A. Our identification of ms^2t^6A is not based upon a known mobility, but rather due to the fact that it exists in $tRNA_5^{Lys}$ from rabbit liver (37).

Nucleotides in positions 34 and 37 were characterized further by chromatography in a two-dimensional solvent system as pNs (Fig. 25). The nucleotide in position 34 is thought to be the hypermodified mcm^5s^2U. Mobilities of this modified nucleotide have not been reported for the various solvent systems used for sequencing studies. It appears just to the right of pG (Fig. 25A). If this specific nucleotide is not present in lymphoma tRNA, I feel that the nucleotide occupying position 34 is nevertheless a hypermodified uridine closely resembling mcm^5s^2U.

Position 37 also is a nucleotide that has not been identified in any of the systems used for "readout sequencing." Its mobilities do not preclude identification as ms^2t^6A.

As in $tRNA_5^{Lys}$, position 34 of $tRNA_{5a}^{Lys}$ probably contains mcm^5s^2U. The two autoradiograms shown in Fig. 26 illustrate the relative mobilities of presumptive mcm^5s^2U in different two-dimensional solvent systems. There is an unknown spot to the right of pG in Fig. 26A so that the result compares favorably to Fig. 25A. From this evidence, I can state that the nucleotide in position 34 of $tRNA_5^{Lys}$ also is present in the same position in $tRNA_{5a}^{Lys}$. In a second two-dimensional system (Fig. 26B), the nucleotide migrates just to the left of pG.

Fig. 25. Two-dimensional chromatogram for identification of positions 34 and 37 of $tRNA_5^{Lys}$. The solutions remaining from positions 34 and 37 after the two one-dimensional TLC runs were treated as stated in Fig. 10. Both positions were chromatographed in solvent system 1. Fig. 25A shows the relative mobility of the presumptive mcm^5s^2U, the spot to the right of pG. Fig. 25B shows a spot most likely representing pt^6Ap and not pt^6A. It was determined that the concentration of nuclease P_1 was too low to digest the hypermodified adenosine.


tRNA$^{Lys}_{S}$
Psn 37
S
B
pA
pG
pUm
pC
pT
pU
pΨ
1
2

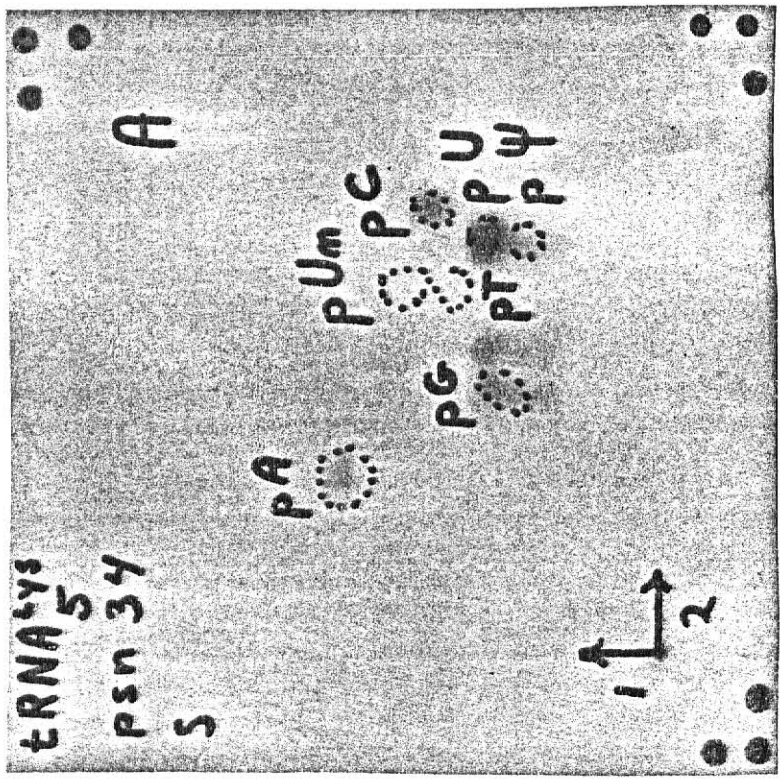
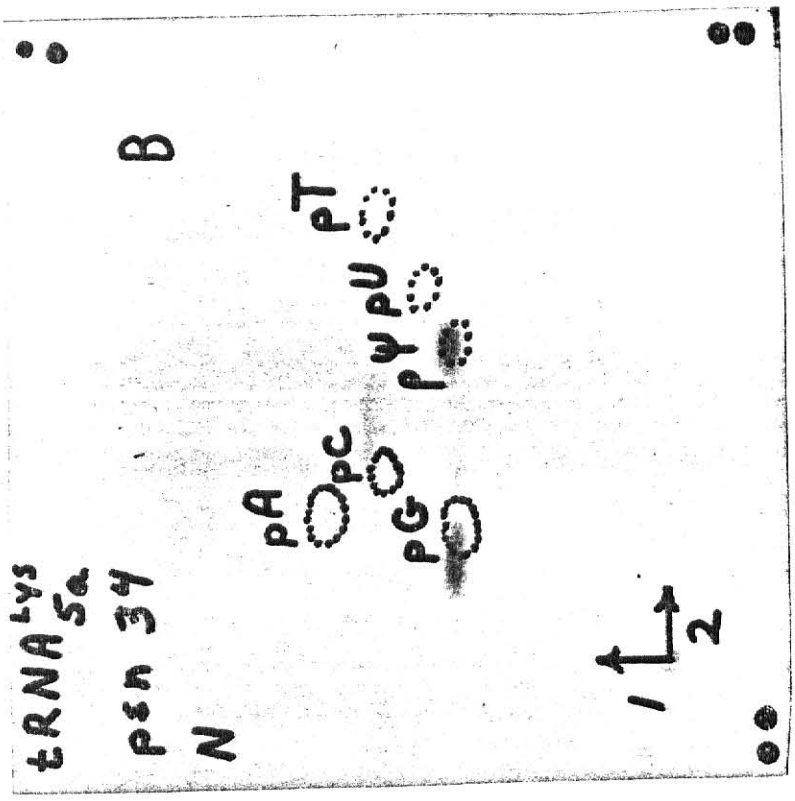
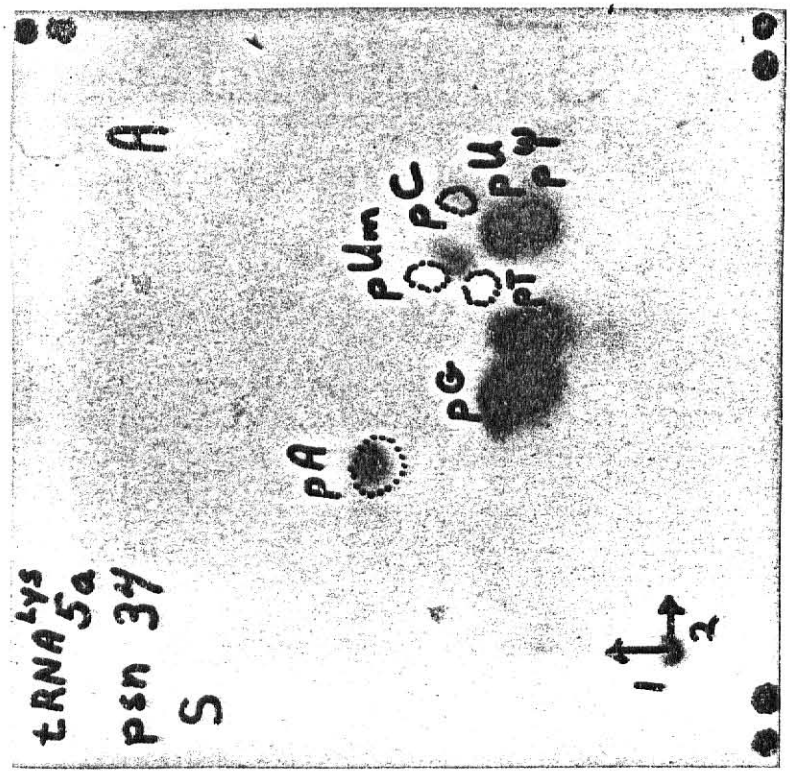

tRNA$^{Lys}_{S}$
Psn 34
S
A
pA
pG
pUm
pC
pT
pU
pΨ
1
2

Fig. 26. Two-dimensional chromatogram for identification of position 34 of $tRNA_{5a}^{Lys}$. The treatment of position 34 for $tRNA_{5a}^{Lys}$ was identical to the treatment for position 36 in $tRNA_{2}^{Lys}$. The P_1 digestion product was chromatographed in solvent system 1 and solvent system 2. The results from the chromatogram from solvent system 1 are shown in Fig. 26A. The relative mobility of this nucleotide is identical to that of the corresponding position in $tRNA_{5}^{Lys}$. The autoradiogram in Fig. 26B shows the results obtained from the two-dimensional chromatogram of position 34 in solvent system 2.


tRNA Lys 5a
psn 34
N
B
pA
pC
pG
pΨ
pU
pT
1
2


tRNA Lys 5a
psn 34
S
A
pA
pG
pUm
pC
pT
pU
pΨ
1
2

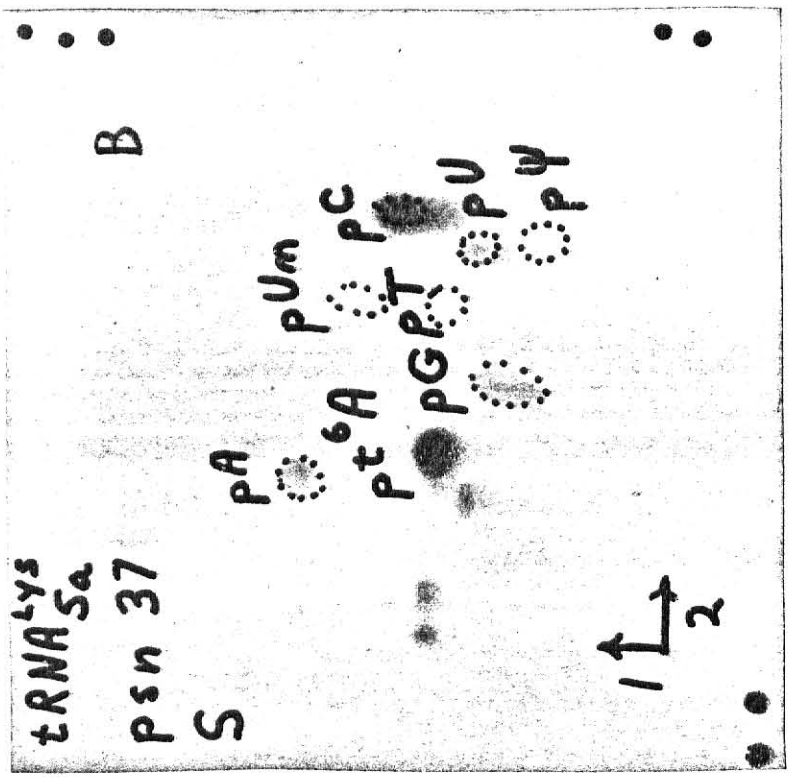
A problem encountered in using the recommended concentration of nuclease P_1 to desphosphorylate certain pNp's to pN's is illustrated in Fig. 27, which displays the relative mobility of the nucleotide present in position 37 in $tRNA_{5a}^{Lys}$ after treatment with a different concentration of nuclease P_1. The mobility obtained for pt^6A (Fig. 27A) does not agree with the mobility described by Silberklang et al. (52). I retreated this nucleotide with 4-fold more nuclease P_1 and rechromatographed the product. The results are displayed in Fig. 27B. The relative mobility of the resulting spot agrees with the mobility expected for pt^6A.

A two-dimensional chromatogram of pTmΨp after digestion with nuclease P_1 is shown in Fig. 28A. The mobility of the spot should have corresponded to pTm but did not. As for t^6A, an increased amount of nuclease P_1 hydrolyzed pTmΨp, giving a spot where pTm should be (Fig. 28B).

The total nucleoside composition of $tRNA_5^{Lys}$ is shown in Fig. 29 and of $tRNA_{5a}^{Lys}$ in Fig. 30. The results are similar with one exception. For $tRNA_{5a}^{Lys}$, the nucleoside t^6A is found, whereas in the autoradiogram for $tRNA_5^{Lys}$ no spot related to t^6A or any modification of t^6A is found. The minor nucleoside present in position 37 of $tRNA_5^{Lys}$ either chromatographed with a major nucleoside or was chromatographed off onto the wick. As stated before, I feel that the hypermodified nucleotide present in position 34 of $tRNA_5^{Lys}$ is ms^2t^6A.

The methods used for sequencing $tRNA_2^{Lys}$, $tRNA_4^{Lys}$, $tRNA_5^{Lys}$, and $tRNA_{5a}^{Lys}$ indicate that two modified nucleotides exist at positions 54 and 55. Data support the identification of the nucleotide at position 54 as 2'-O-methyl-ribothymidine. The presence of the 2'-O-methyl group precludes direct identification of the nucleotide in 55 because formamide hydrolysis, or

Fig. 27. Two-dimensional chromatogram for identification of position 37 of $tRNA_{5a}^{Lys}$. The nucleotide was treated as in Fig. 10. As indicated by the results in Fig. 27A the concentration of nuclease P_1 was not sufficient to convert pt^6Ap to pt^6A. The nucleotide was retreated by increasing the P_1 concentration four-fold. The P_1 digest was rechromatographed in solvent system 1. The results shown in Fig. 27B indicate that the increase in P_1 was sufficient to convert pt^6Ap to pt^6A.


B
tRNA$^{Lys}_{Sa}$
Psn 37
S
pA
pt^{6}A
pG
pT
pUm
pC
pU
pΨ
1
2

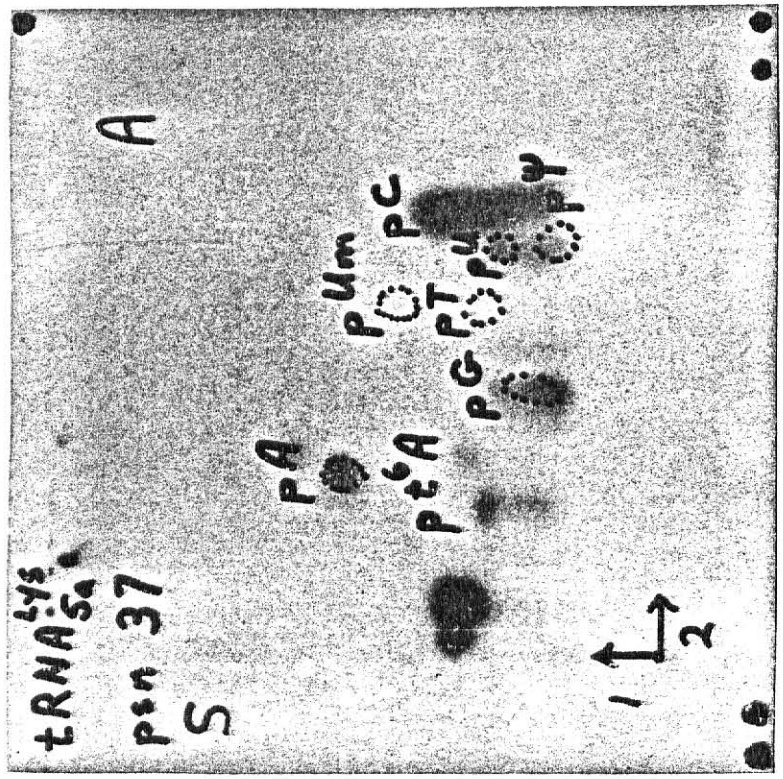
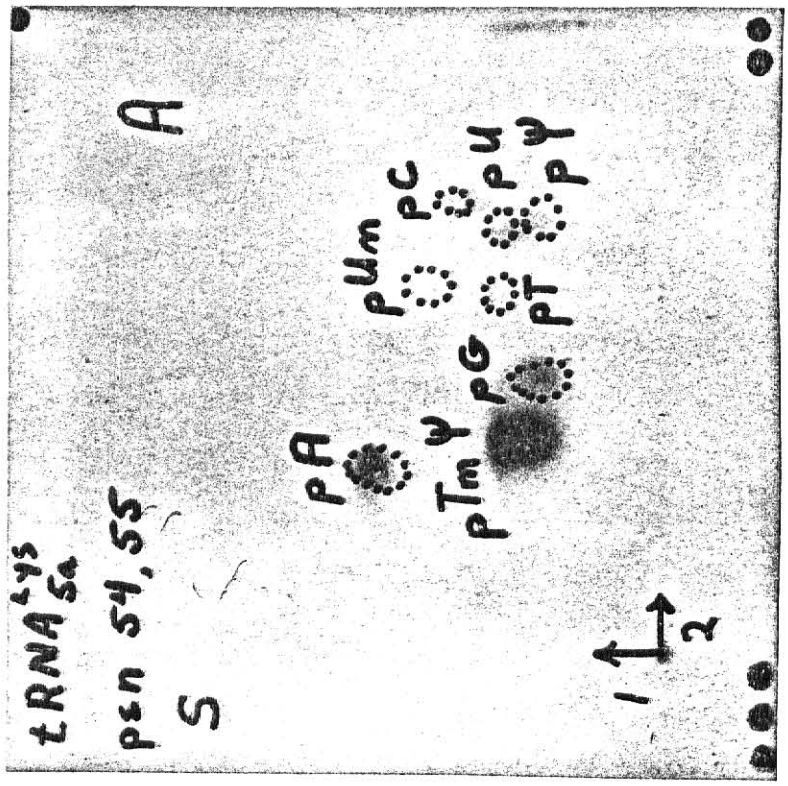

A
tRNA$^{Lys}_{Sa}$
Psn 37
S
pA
pt^{6}A
pG
pT
pUm
pC
pU
pΨ
1
2

Fig. 28. Two-dimensional chromatogram for identification of positions 54-55 of $tRNA_{5a}^{Lys}$. The dinucleotide TmΨ in positions 54-55 was treated as the nucleotide in Fig. 27A. The result is shown in Fig. 28A. Again, the P_1 concentration was too low to provide any significant cleavage of the TmΨ linkage. The dinucleotide was retreated with P_1 as in Fig. 27B. The results shown in Fig. 28B indicate the presence of pTm, the nucleotide expected upon cleavage of the TmΨ linkage.


A
tRNA$^{Lys}_{Sa}$
Psn 54, 55
S
pA
pTm
Ψ
pG
pT
pUm
pC
pU
pΨ
1
2

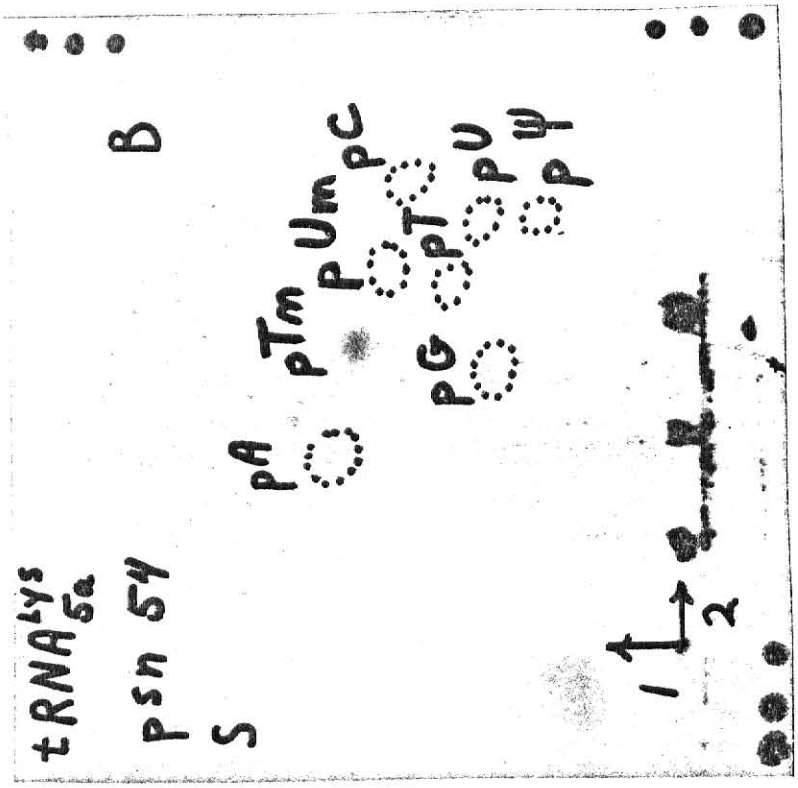

B
tRNA$^{Lys}_{Sa}$
Psn 54
S
pA
pTm
pUm
pC
pG
pT
pU
pΨ
1
2

Fig. 29. Total nucleoside composition of $tRNA_5^{Lys}$. The procedure followed was that of Randerath et al. (69)(see Experimental Procedures). All of the nucleotides found in the "sequencing readout" are present in the total nucleoside composition of $tRNA_5^{Lys}$ with the exception of ms^2t^6A. It is thought that this either chromatographed with a major nucleoside or ran off onto the wick.

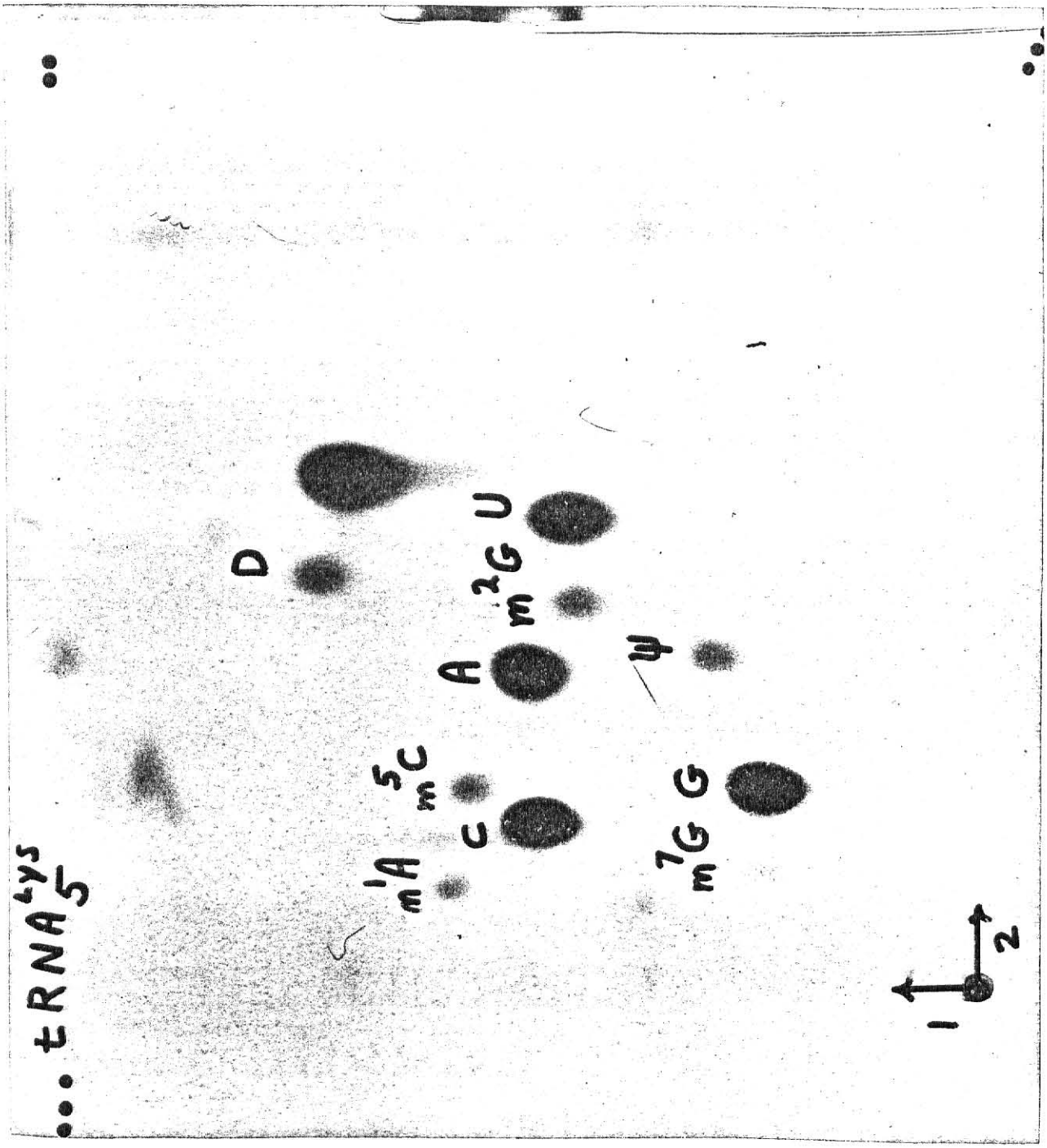

... tRNA$^{Lys}_{S}$
m^1A
m^5C
C
A
D
m^2G
U
Ψ
m^7G
G
1
2

Fig. 30. Total nucleoside composition of $tRNA_{5a}^{Lys}$. The procedure followed was that of Randerath et al. (69)(see Experimental Procedures). The nucleoside content of $tRNA_{5a}^{Lys}$ differs from $tRNA_{5}^{Lys}$ by the appearance of t^6A. This was consistent with the results from the "sequencing readouts."

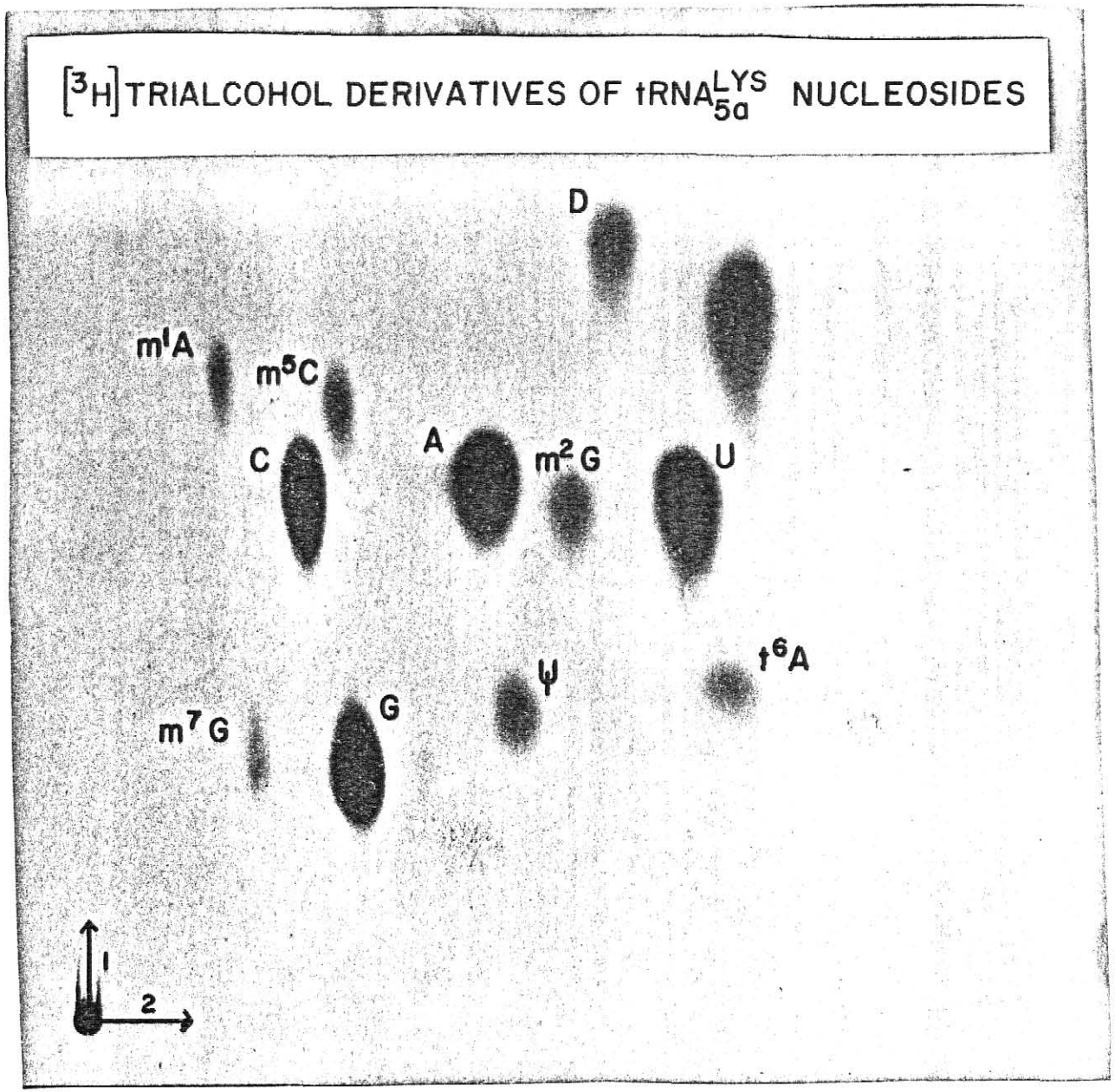

[3H] TRIALCOHOL DERIVATIVES OF tRNA 5a LYS NUCLEOSIDES
D
m1A
m5C
C
A
m2G
U
t6A
ψ
m7G
G
1
2

TABLE 3

SUMMARY OF SEQUENCING RESULTS FOR $TRNA_5^{LYS}$

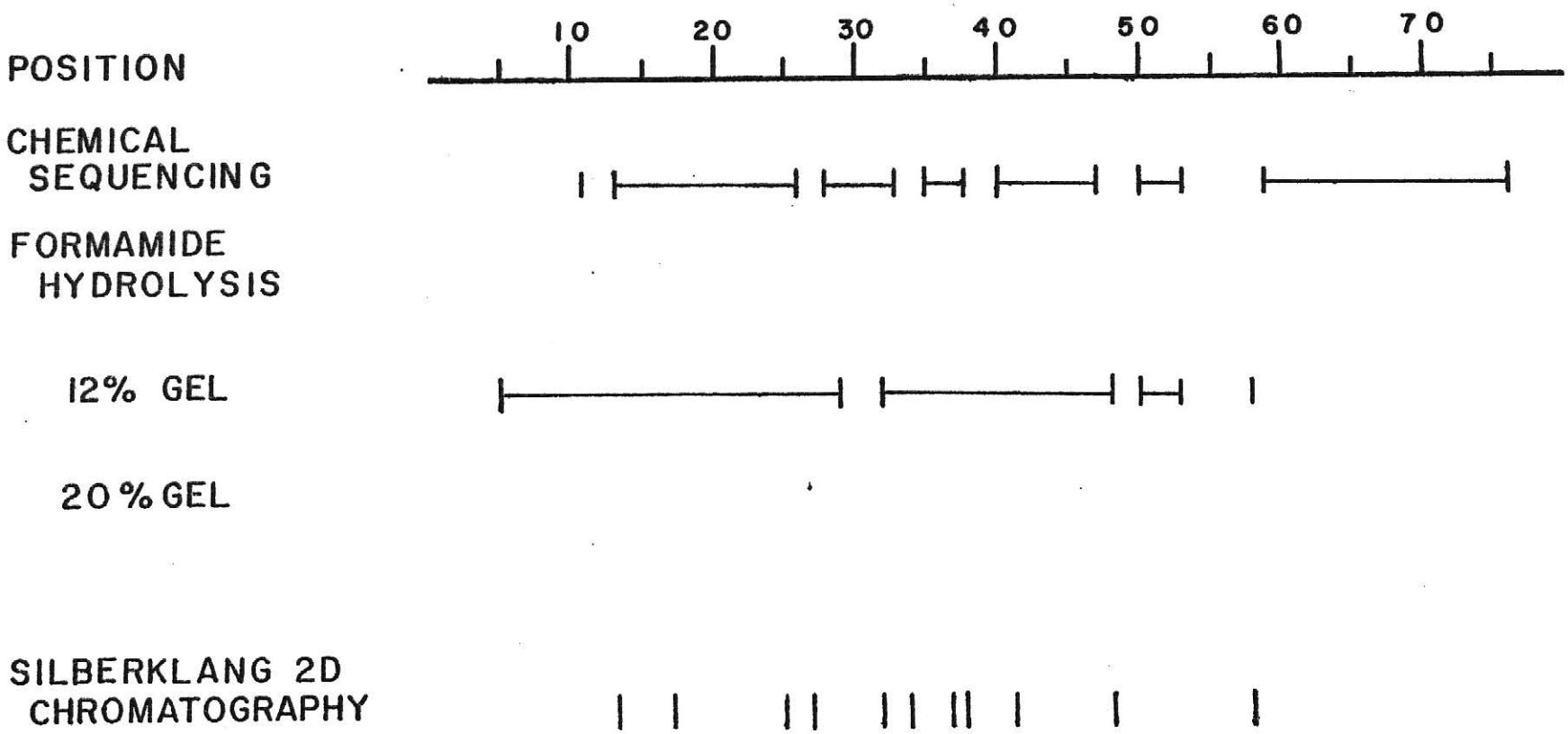


TABLE 4

SUMMARY OF SEQUENCING RESULTS FOR $TRNA_{5a}^{LYS}$

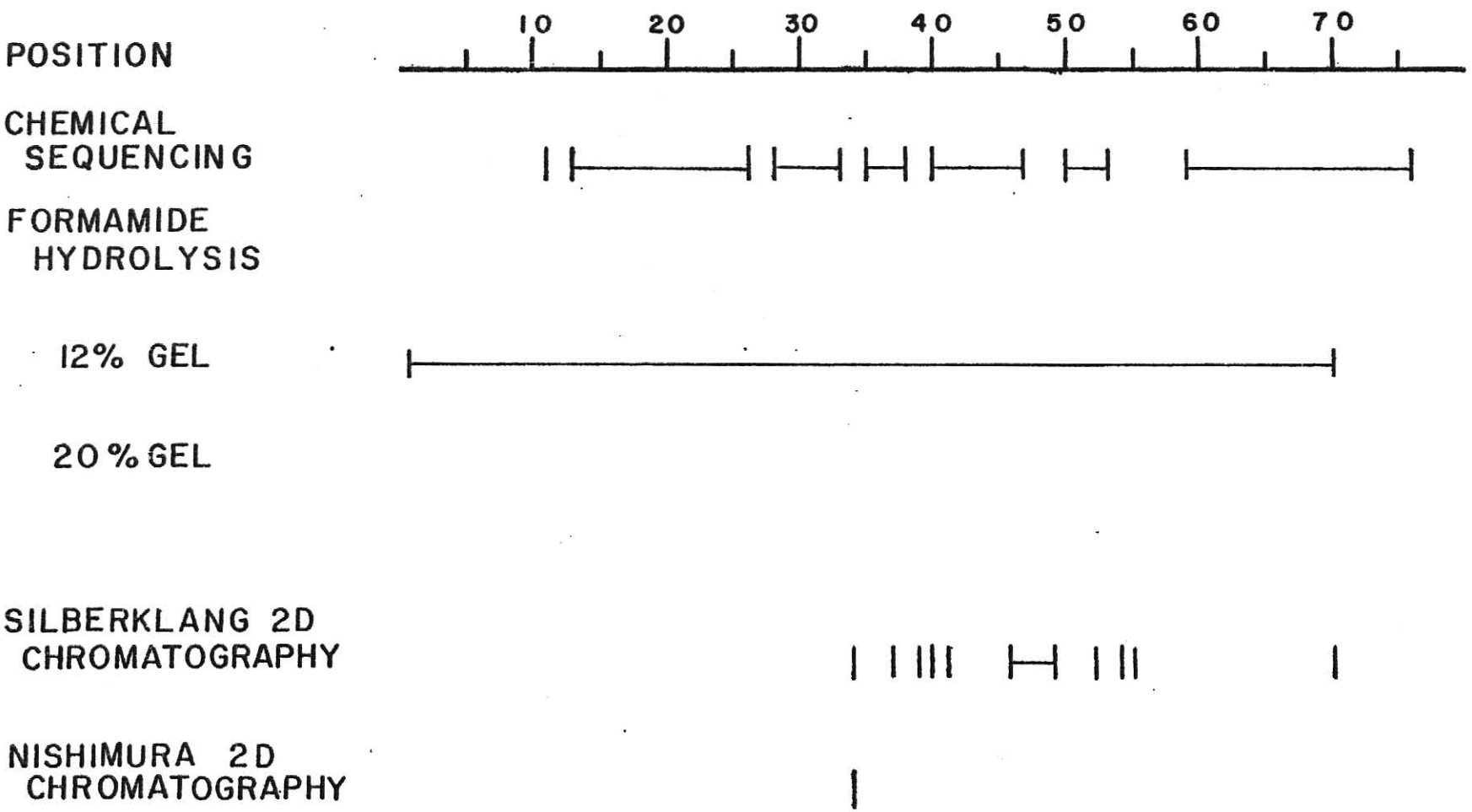


Fig. 31. Nucleotide sequence of $tRNA_5^{Lys}$.

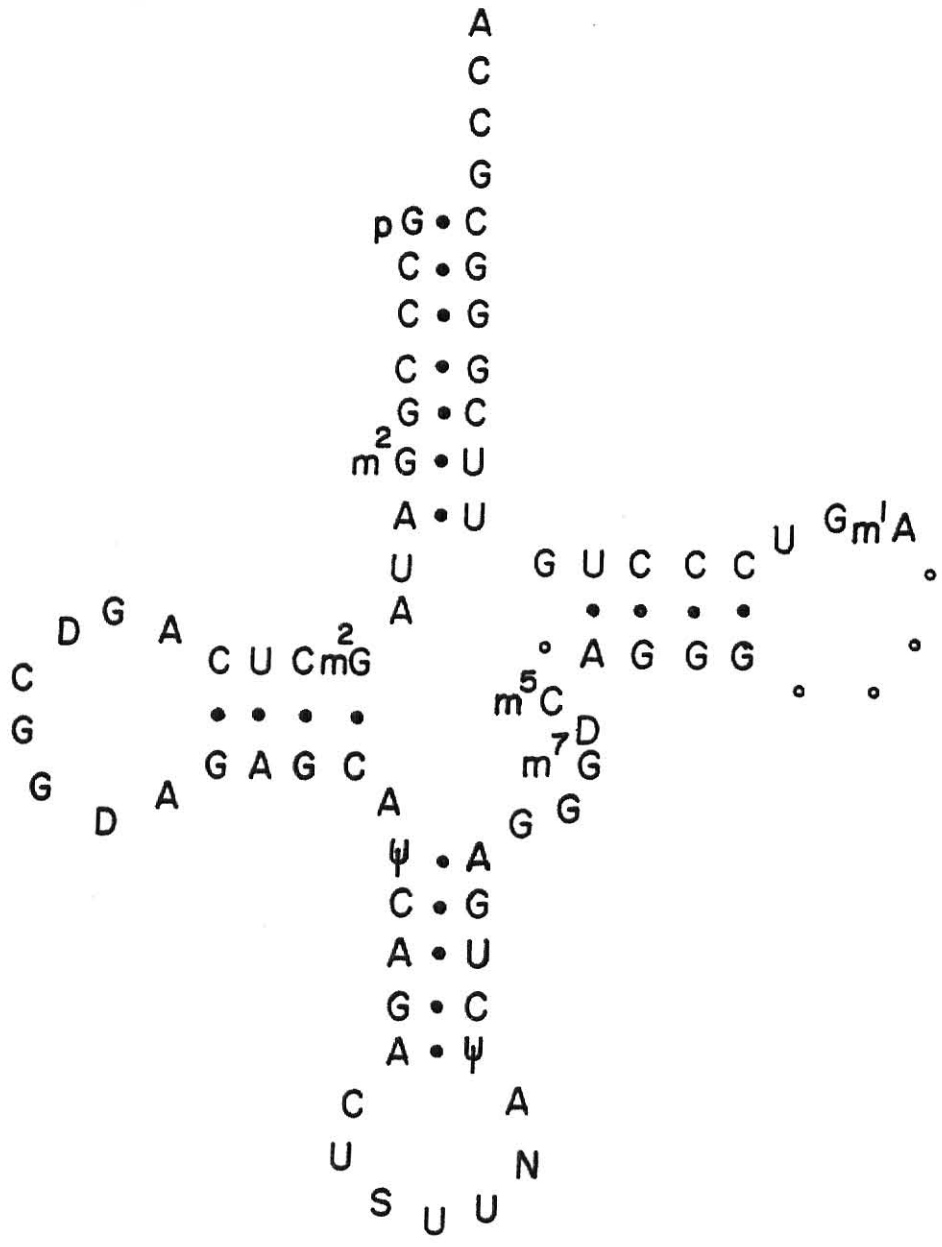
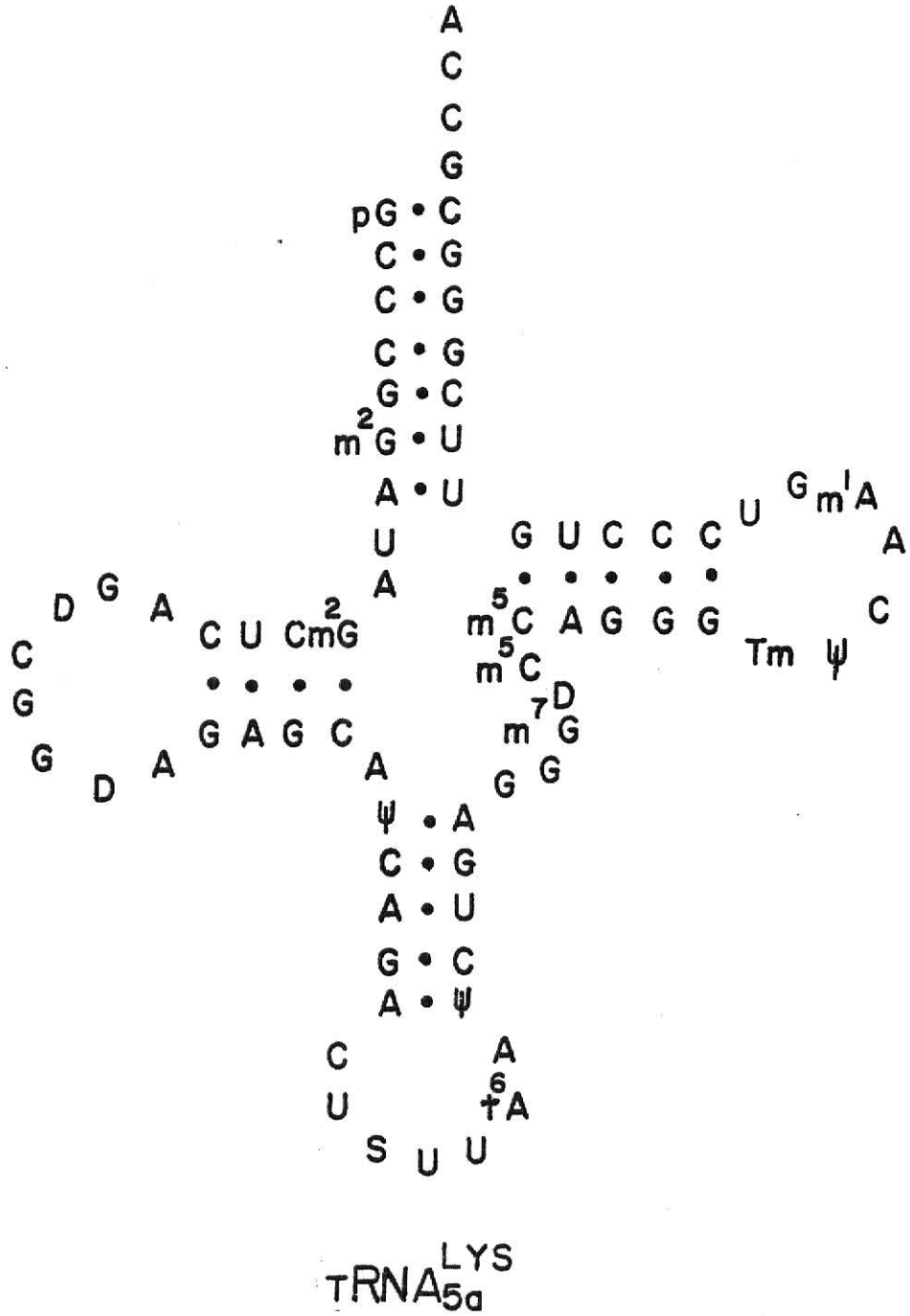

A
C
C
G
pG • C
C • G
C • G
C • G
G • C
m2G • U
A • U
U
A
G U C C C U GmIA
A G G G
m5C
m7G
G G
D G A
C U C mG2
G A G C
D A A
ψ • A
C • G
A • U
G • C
A • ψ
C A
U N
S U U
TRNA5LYS

Fig. 32. Nucleotide sequence of $tRNA^{Lys}_{5a-}$.

Fig. 32. Nucleotide sequence of $tRNA^{Lys}_{5a}$.


A
C
C
G
pG • C
C • G
C • G
C • G
G • C
m2G • U
A • U
U
A
G U C C C
U
G m1A
A
D G A
C U Cm2G
m5C A G G G
C
C
G
m5C
Tm ψ
m7D
G A G C
m7G
G
A
A
G G
D
ψ • A
C • G
A • U
G • C
A • ψ
C
A
U
6
tA
S U U
TRNALYS
5a

alkaline hydrolysis of any form, does not cleave between positions 54 and 55. Thus, the assignment of position 55 as pseudouridine is based on the presence of 3 residues of pseudouridine in each molecule of tRNA as determined from the nucleoside composition and because chemical sequencing would have indicated the presence of an unmodified uridine at that position.

Sequence of $tRNA_6^{Lys}$ from Balb/3T3 cells. The sequence of major nucleotides of $tRNA_6^{Lys}$ was determined by the enzymatic sequencing method. The method is very similar to chemical sequencing in that RNases make specific cuts. Under the correct conditions, the treatment generates a nested set of fragments which is resolved on polyacrylamide gels under denaturing conditions. The sequence is read directly from an autoradiogram of the gel.

This procedure gave the sequence of major nucleotides from nucleotide 14 through nucleotide 75. It is not apparent why nucleotides 15, 32, and 33 could not be identified. The other positions 34, 37, and 46 can not be identified from the gel because the enzymes do not cleave at modified nucleotides. A band for position 76 is not present because the terminal adenosine was run off the gel.

$tRNA_6^{Lys}$ was subjected to formamide hydrolysis to produce the ladder displayed in Fig. 34, and bands were excised and treated as described in Experimental Procedures. With this technique I determined the sequence from position 1 through position 73. Autoradiograms of thin layer chromatograms illustrating partial sequence information are shown in Fig. 35 and 36.

Positions 1 through 15 are identified in Fig. 35. There is no evidence for the m^2G found in positions 6 and 10 as in the other isoacceptors. In

Fig. 33. Sequencing ladder from enzymatic hydrolysis of $tRNA_6^{Lys}$. Each lane represents an enzyme reaction, where the RNase type is signified by the upper set of letters. A control lane signified by Co and a formamide hydrolysis lane signified by F are also present. The enzyme nucleotide specificities are shown in the lower set of letters. Each lane contains 3,000 cpm of [5'-^{32}P]pCp-labeled tRNA. Three additions were made to the gel. The second addition was added when the xylene cyanol marker of the first addition was 22 cm from the origin. The third addition was made when the xylene cyanol FF dye of addition two was at 16.5 cm from the origin. Each band is labeled to indicate the nucleotide identified. The sequence is read from the bottom up, starting with addition 2 (right hand set of lanes). When reading of addition 2 becomes difficult due to lack of resolution, addition 1 is then used. The overlap of the two additions can be located by the xylene cyanol FF marker (XC). The autoradiogram was exposed for 19 hr at -70° C with an intensifying screen.

$tRNA_6^{Lys}$

Enzyme Sequencing

Fig. 34. Sequencing ladder from formamide hydrolysis of $tRNA_6^{Lys}$. The procedure followed for the production of this ladder was the same as that for Fig. 7. The autoradiogram was exposed for 1.5 hr at -70° C with an intensifying screen.

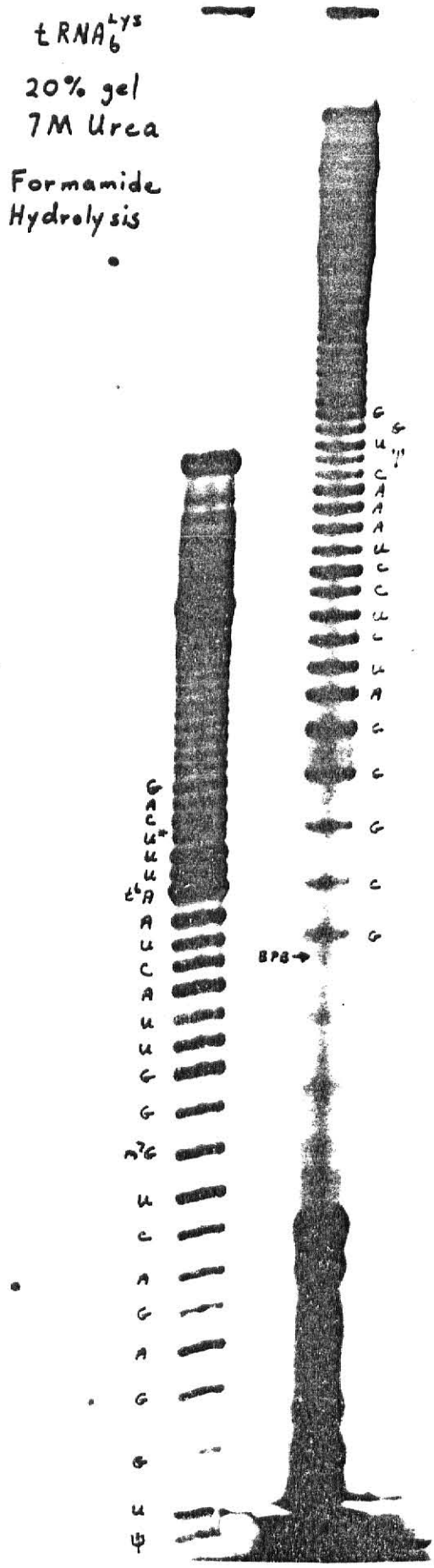
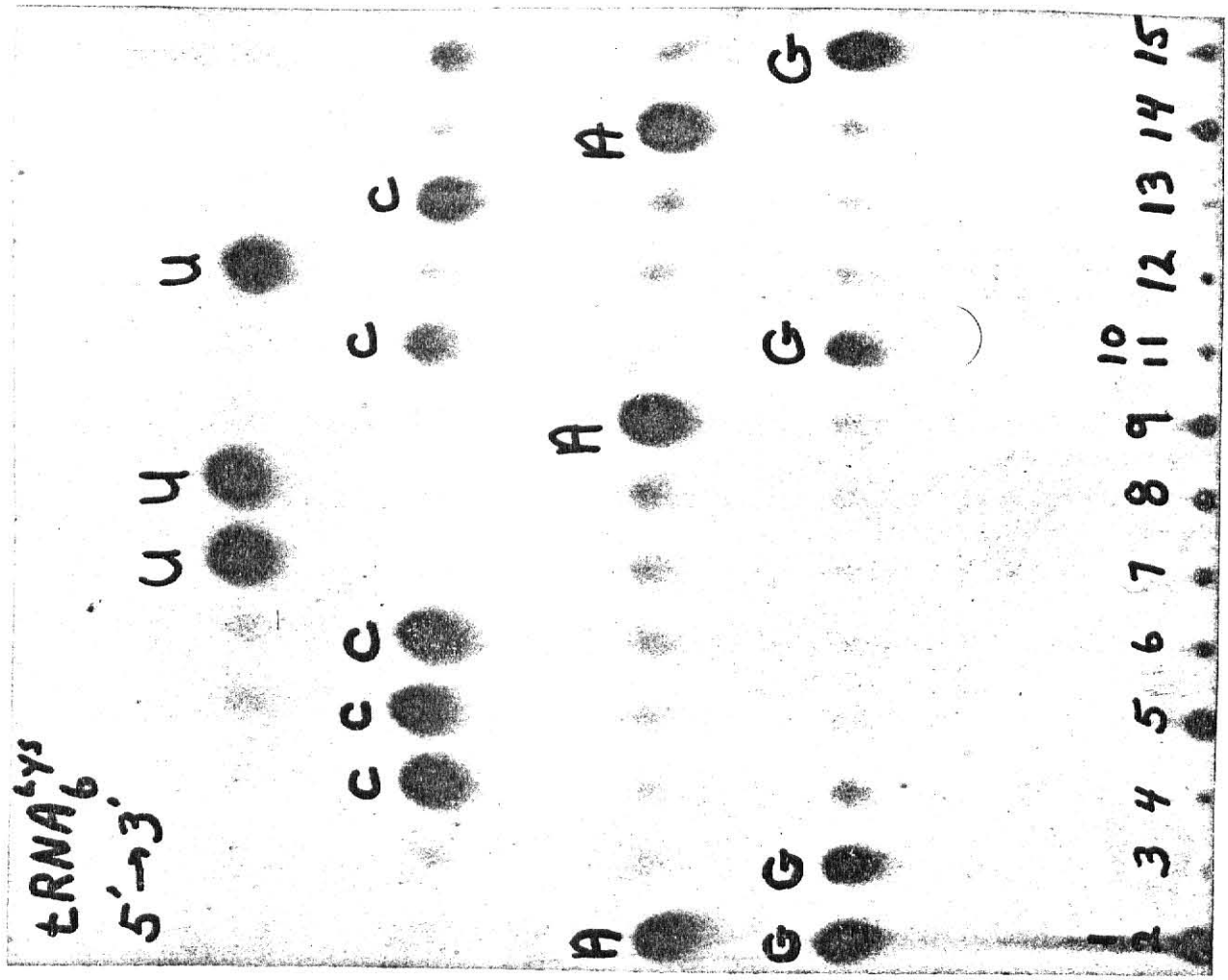

tRNA$_6^{Lys}$
20% gel
7M Urea
Formamide
Hydrolysis
BPB
G A C U* U U U A A U C A U U C G G m^{2}G U C A G A G G U Ψ
G Ψ U C A A A U C C U U A A C C G C G
G U C A A A U C C U U A A C C G C G

Fig. 35. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_6^{Lys}$ (nucleotides 1-15). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. Positions 1-2 and 10-11 are each contained in one lane. This is due to the phenomenon of band compression. The absence of m^2G in positions 6 and 10 should be noted.


tRNA$^{Lys}_{6}$
5'→3'
A
G G
C C C
U U
C C
A A
G G
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

fact, there are no minor nucleotides in the structure until position 34, which is the 5' side of the anticodon. Results covering the anticodon, positions 34-36, are shown in Fig. 36. Position 34 is the minor nucleotide U*, the structure of which is unknown (70, 71). Position 36 also contains a minor nucleotide of unknown structure, which migrates to a location that differs from both U and U*. This nucleotide was designated U**. Position 37 is the hypermodified adenosine, t^6A.

The m^7G found in position 46 of the other isoacceptors presented in this work also is present in this tRNA. The highly conserved GTΨC tetranucleotide is found to be undermodified in this structure. Rather than ribothymidine or a 2'-O-methylribothymidine in position 54, uridine is present.

To confirm assignments, nucleotides 34, 37, and 46 were treated with nuclease P_1 and chromatographed in a two-dimensional system. The results are shown in Fig. 37 and 38.

The mobility of the major nucleotide for position 34 in the chromatographic system used in Fig. 37A is that of U* (70, 71).

As in $tRNA_2^{Lys}$, the pt^6Ap is not completely converted to pt^6A by nuclease P_1 (Fig. 37B), but the results confirm that t^6A is in position 37.

The assignment, from results of one dimensional chromatograms, of position 46 as m^7G was not definitive, but treatment of the nucleotide with nuclease P_1 and two-dimensional chromatography clearly produced results establishing position 46 as m^7G (Fig. 38).

The lack of modification found in $tRNA_6^{Lys}$ was confirmed by determining the total nucleoside composition (69). Results are shown in Fig. 39 and indicate that there are four identifiable modified nucleosides: m^7G, D, m^5C, and t^6A. Neither D nor m^5C were found by sequencing studies. There

Fig. 36. Autoradiogram of a PEI-cellulose chromatogram for "sequencing readout" of $tRNA_6^{Lys}$ (nucleotides 19-37). The procedure followed for producing this "sequencing readout" is the same as that for Fig. 8. The relative mobilities for the modified nucleotides U*, U** and t^6A are shown in this readout.

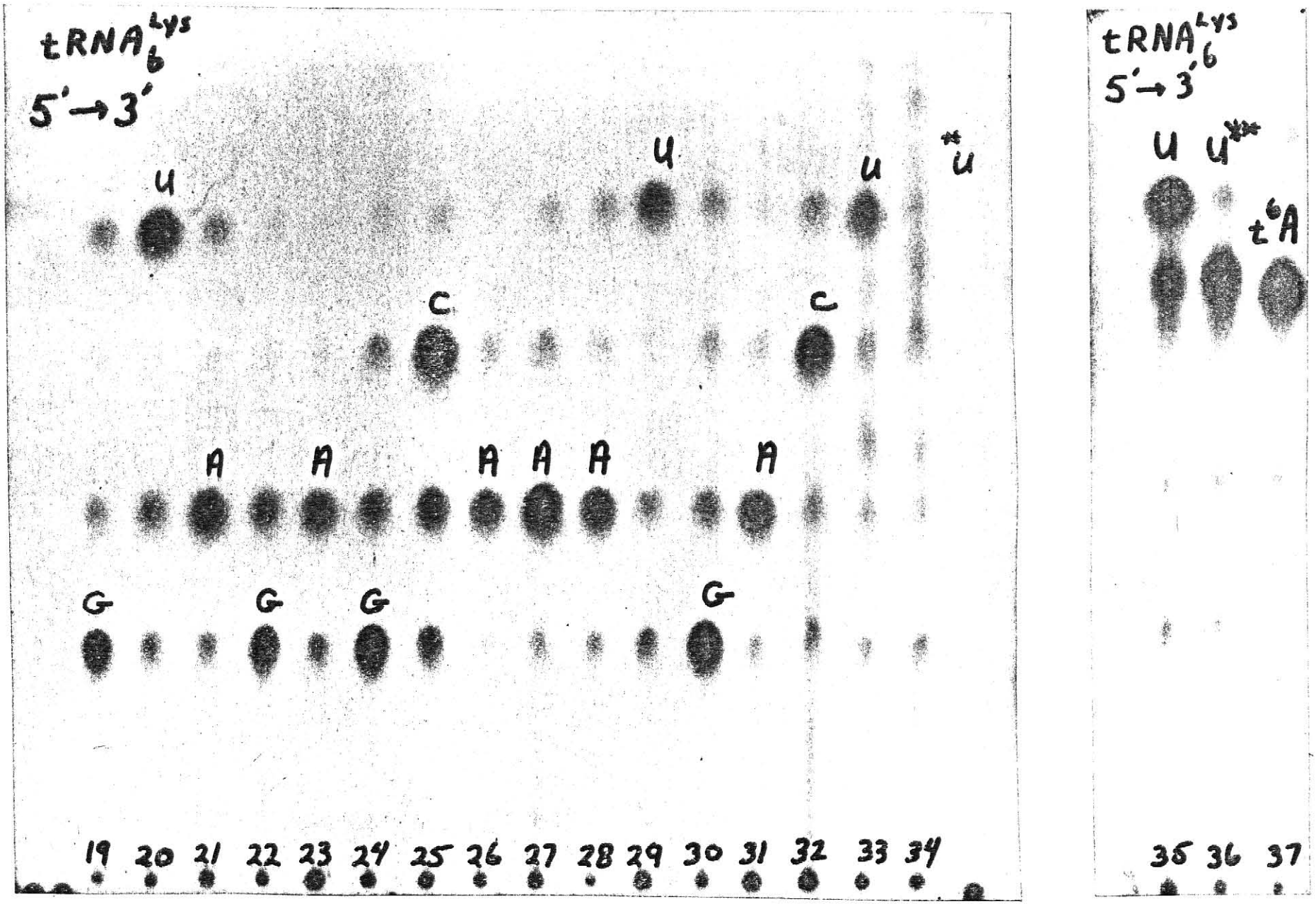
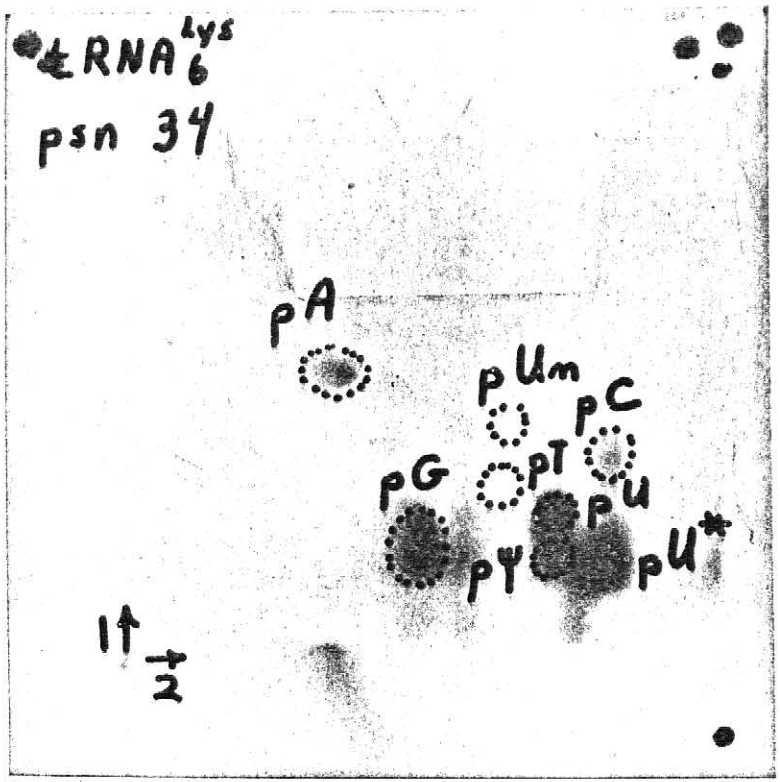

tRNA$^{Lys}_{6}$
5'→3'
U
U
U
*U
C
C
A
A
A
A
A
A
G
G
G
G
19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
tRNA$^{Lys}_{6}$
5'→3'
U
U**
t^{6}A
35 36 37

Fig. 37. Two-dimensional chromatogram for identification of positions 34 and 37 from $tRNA_6^{Lys}$. Each nucleotide was treated as in Fig. 10. Fig. 37A shows the relative mobility of U* in solvent system 1. Fig. 37B confirms the presence of t^6A in position 37. Note that there is still a large amount of the pt^6Ap (far left hand spot) left after P_1 digestion.


tRNA$^{Lys}_6$
psn 34
pA
pUm
pC
pG
pT
pU
pΨ
pU*
1
2

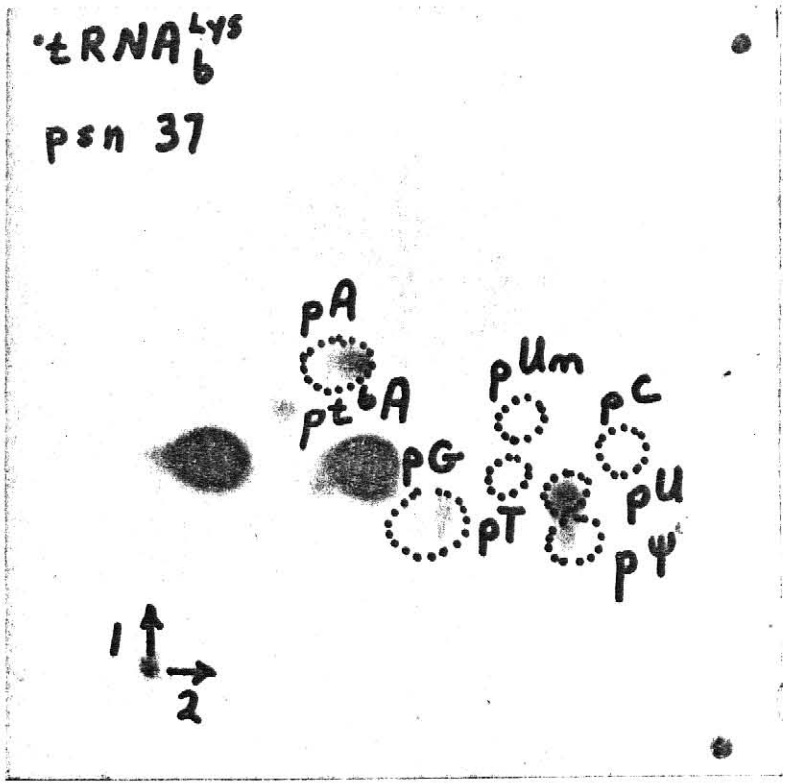

tRNA$^{Lys}_6$
psn 37
pA
ptA
pUm
pC
pG
pT
pU
pΨ
1
2

Fig. 38. Two-dimensional chromatogram for identification of position 46 of $tRNA_6^{Lys}$. The nucleotide was treated as stated in Fig. 10. The presence of m^7G in position 46 is confirmed by this autoradiogram.

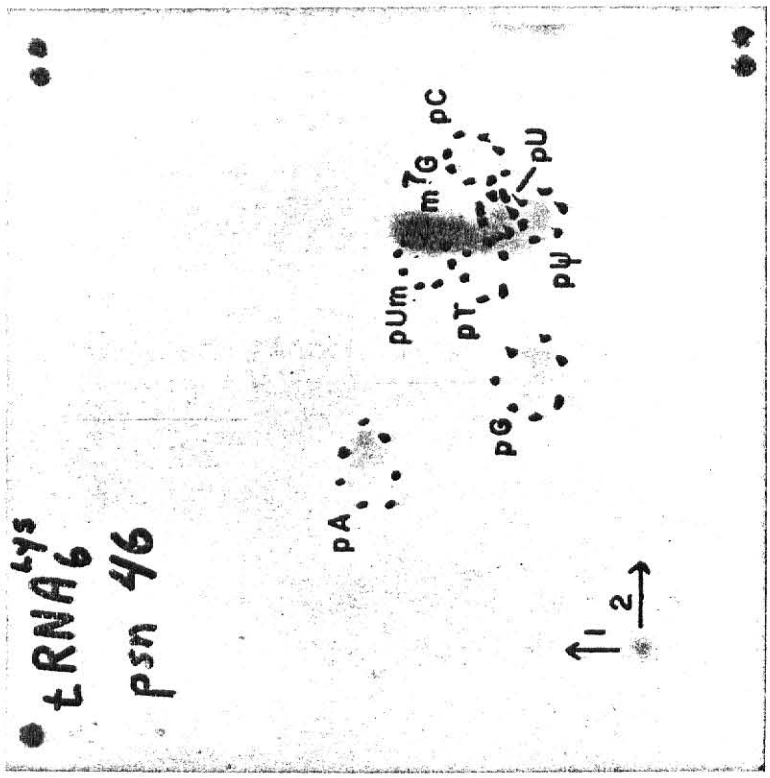
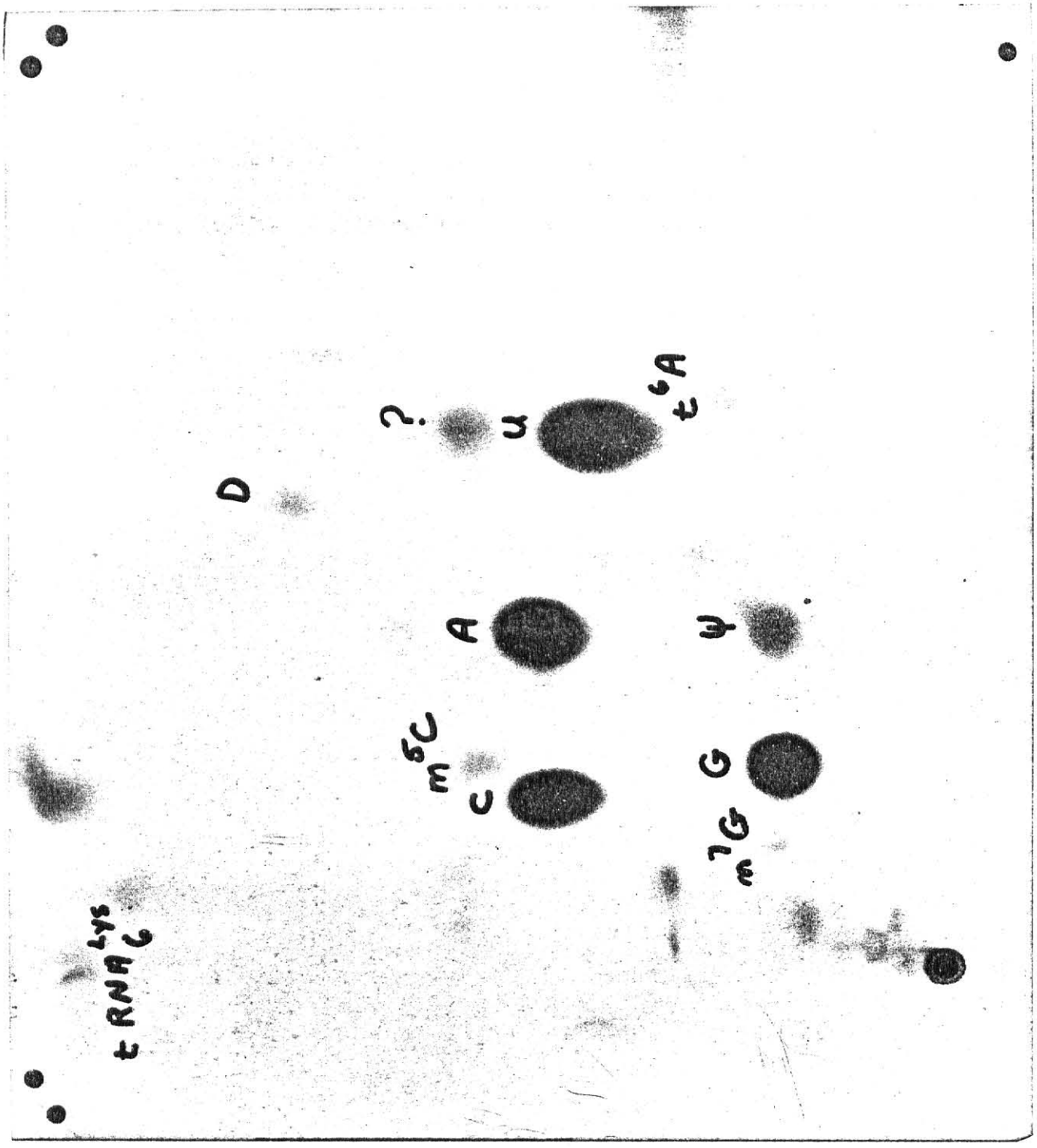

tRNA$^{Lys}_6$
Psn 4/6
pA
pG
pT
pUm
m^{7}G
pC
pU
pψ
1
2

Fig. 39. Total nucleoside composition of $tRNA_6^{Lys}$. The procedure followed was that of Randerath et al. (69) (see Experimental Procedures). The deficiency of modified nucleotides found in the "sequence readouts" is confirmed by this autoradiogram. The presence of dihydrouridine and m^5C are due to contamination. The spot just above U is an unidentified nucleoside. It is thought that this may be either U* or U**.


tRNA$^{Lys}_{G}$
m^{5}C
C
A
D
?
U
t^{6}A
Ψ
m^{7}G
G

appears to be another unidentifiable nucleoside above U. It is possible that the modification above U is U* or perhaps the U**.

The sequencing approaches used with $tRNA_6^{Lys}$ are summarized in Table 5. The nucleotide sequence of $tRNA_6^{Lys}$ is shown in cloverleaf form in Fig. 40.

TABLE 5

SUMMARY OF SEQUENCING RESULTS FOR $TRNA_6^{LYS}$

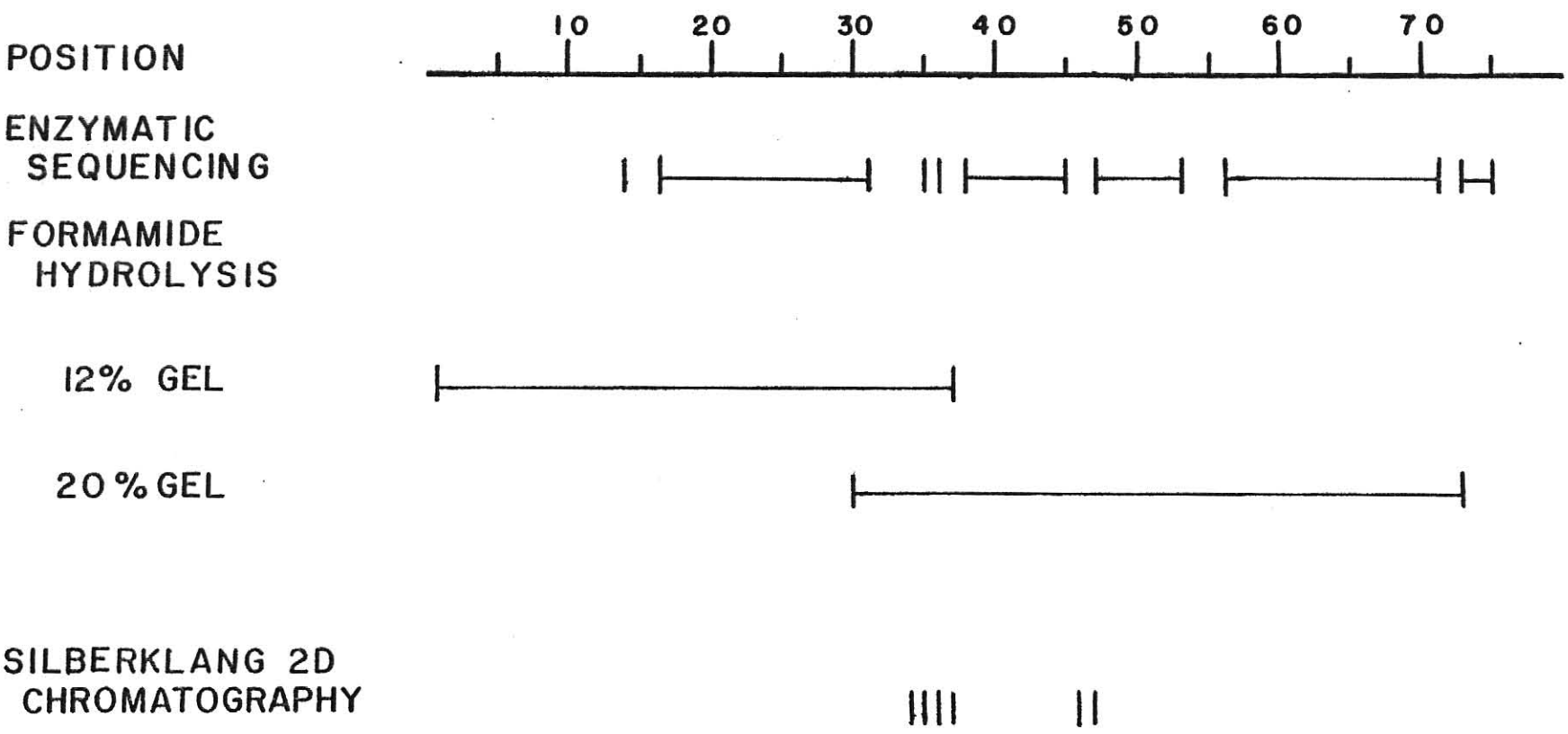
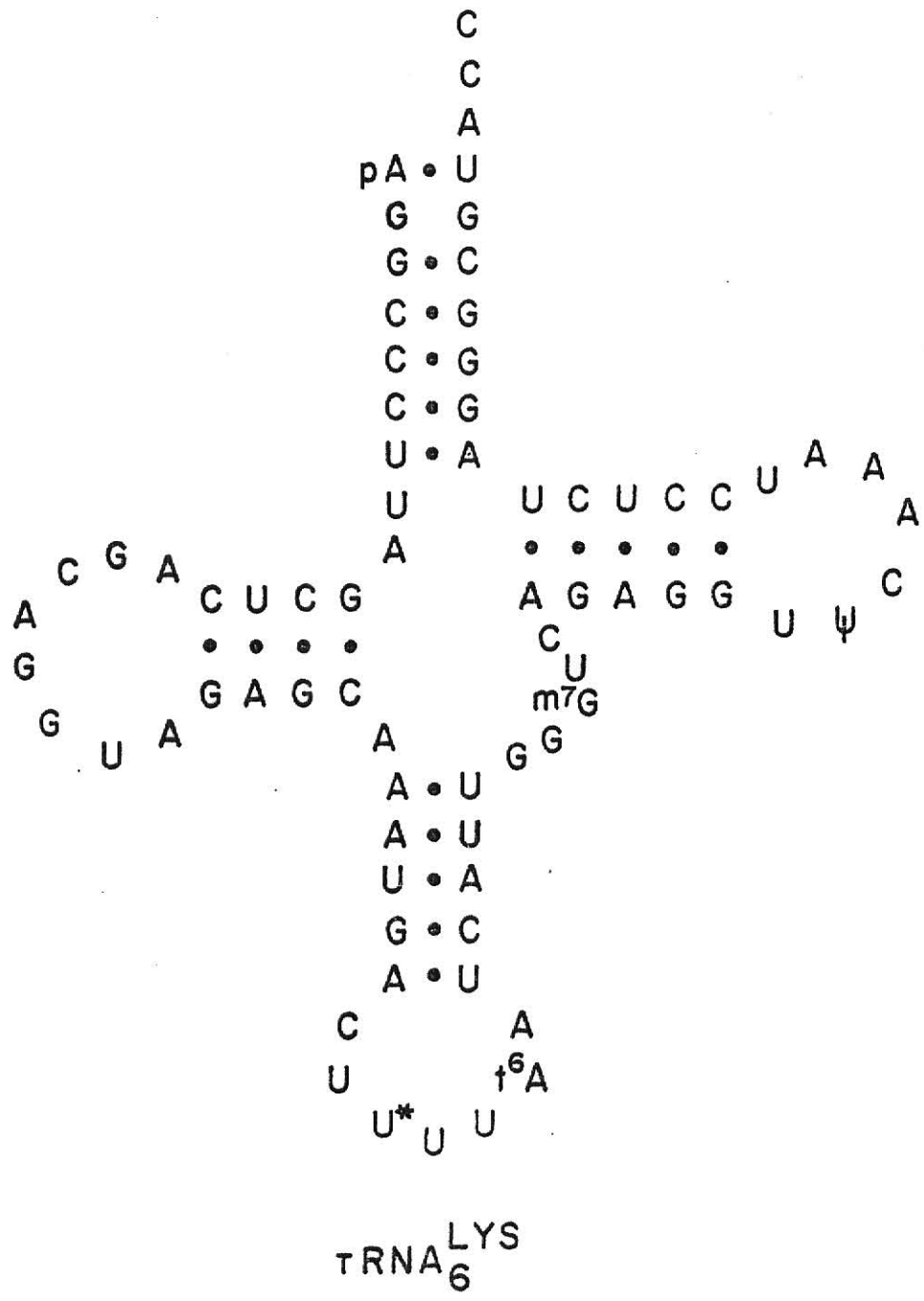


Fig. 40. Nucleotide sequence for $tRNA_6^{Lys}$.


C
C
A
pA • U
G G
G • C
C • G
C • G
C • G
U • A
U
A
U C U C C
A G A G G
C
U
m7G
G G
A • U
A • U
U • A
G • C
A • U
C A
U t6A
U* U U
C G A
C U C G
G A G C
G U A
A
G
G
U A A
A
C
U ψ
tRNA LYS 6

DISCUSSION

The research reported in this thesis provides nucleotide sequences for five lysine tRNAs and the major nucleotide sequences for two other lysine tRNAs. Nothing was known about the sequences of two of these tRNAs, $tRNA_{5a}^{Lys}$ and $tRNA_{6}^{Lys}$, before the data presented here were collected. Furthermore, with regard to the other sequences, there are some interesting points of difference with a previous report (37).

$tRNA_2^{Lys}$ and $tRNA_4^{Lys}$

The sequences obtained in this study differ only slightly from those reported by Raba et al. (37).

I show that m^2G is present in positions 6 and 10 in both species, whereas Raba et al. reported finding it only in position 10. I also found the same modification in $tRNA_{5a}^{Lys}$ and $tRNA_{5}^{Lys}$. Because m^2G was found in position 6 in the four tRNAs, I feel that this is a true representation of this nucleotide and not an artifact. In addition, the guanosine nucleotide which should be in position 6 is not found; thus, it is reasonable that the m^2G replaces G.

Differences with the results of Raba et al. (37) were also found at positions 36, 37, and 54 of $tRNA_4^{Lys}$ and positions 35 and 36 of $tRNA_2^{Lys}$. There is an unidentified nucleotide, probably a modified uridine (which I refer to as U**), occurring in quantities greater than uridine in position 36 of $tRNA_2^{Lys}$ and in quantities less than uridine in position 35 of $tRNA_2^{Lys}$ and 36 of $tRNA_4^{Lys}$. It is not likely that U** is 2'-O-methyluridine because pt^6Ap would not appear in the sequencing data in expected quantities.

In $tRNA_2^{Lys}$, position 37 is represented by the hypermodified nucleotide t^6A. In this position of $tRNA_4^{Lys}$, t^6A is found in only a limited amount. The nucleotide in this location has not been characterized as yet and can not be identified with quantities of material available to us. Raba et al. (37) stated that the nucleotide in position 37 of $tRNA_4^{Lys}$ is probably a closely related form of t^6A or a precursor of t^6A. A precursor to t^6A would have to be a carbamylated adenosine ready to receive the threonine moiety. It has been suggested that the process of converting adenosine to t^6A is accomplished by a single enzyme using ATP, tRNA, HCO_3^{-2}, and threonine as substrates (72). This complex enzyme has the ability to use glycine as a substrate in place of threonine, and serine, with its close structural similarity to threonine, may also be a substrate (72). If this new modified nucleoside contains glycine or serine instead of threonine, the difference between $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ could be explained; however, the precursor role of $tRNA_4^{Lys}$ would not be compatible with such an explanation.

Another hypothesis is that the t^6A in $tRNA_4^{Lys}$ is a species phosphorylated on threonine. The postlabeling method (69) for determining the total nucleoside composition uses a phosphatase which would hydrolyze the phosphate and give the t^6A found in the total nucleoside composition of $tRNA_4^{Lys}$. A phosphatase is not used in the sequencing procedures; thus, a phosphorylated pt^6Ap would have altered chromatographic mobilities. If this phosphate does occur on t^6A, there is the possibility of a regulatory role for $tRNA_4^{Lys}$ operating through phosphorylation-dephosphorylation.

Position 54 is Tm in $tRNA_2^{Lys}$, $tRNA_{5a}^{Lys}$, $tRNA_5^{Lys}$, and $tRNA_4^{Lys}$. Tm in $tRNA_4^{Lys}$ was not identified directly; rather, the dinucleotide TmΨ was identified by comparison with TmΨ from $tRNA_{5a}^{Lys}$ from which Tm was shown to

be a component of a dinucleotide (Fig. 28). Raba et al. (37) reported that $tRNA_4^{Lys}$ from SV40-transformed Balb/3T3 cells has T, Tm, Ψ, and U at position 54.

The minor isoacceptors cochromatographing with $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ differ from these major isoacceptors in the sequence of major nucleotides in the middle base pair of the anticodon stem. In $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ there is an A-U base pair in these positions, whereas in the minor isoacceptors there is a G-C base pair. Although the minor nucleotides of the minor isoacceptors were not determined, it is probable that each of the minor species has a minor nucleotide composition like the major isoacceptor. Results of chemical sequencing show gaps in the sequences for the minor isoacceptors analogous to gaps for the major isoacceptors. Raba et al. (37) reported two isoacceptors which correspond to the $tRNA_2^{Lys}$ and its minor species reported here. They did not find a corresponding minor species for $tRNA_4^{Lys}$. Thus, if $tRNA_4^{Lys}$ is an undermodified form of $tRNA_2^{Lys}$ in all respects including t^6A, lymphoma cells appear to produce an undermodified form of $tRNA_{2\ minor}^{Lys}$, whereas SV40-transformed Balb/3T3 cells do not, although these cells produce a similar undermodified form of $tRNA_2^{Lys}$: $tRNA_4^{Lys}$.

$tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$

The m^2G in position 6 of lymphoma $tRNA_5^{Lys}$ is the only confirmed difference between $tRNA_5^{Lys}$ from lymphoma and rabbit liver (37). Position 34 in rabbit liver $tRNA_5^{Lys}$ is mcm^5s^2U (37). The occurrence of this modified uridine in lymphoma $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ was not confirmed in this study, but the same modified nucleotide occurs in position 34 in both of these tRNAs and is probably mcm^5s^2U.

The sequence for positions 1 through 4 of $tRNA_5^{Lys}$ was not established, but it is undoubtedly the same as in lymphoma $tRNA_{5a}^{Lys}$ and rabbit liver $tRNA_5^{Lys}$. The same argument can be applied to positions 54-57.

This thesis is the first report of a sequence for $tRNA_{5a}^{Lys}$. On the basis of its structure, it is a species that is coded within the nucleus, but there is evidence that it is associated with the mitochondria in mouse liver (K. Thomas and C. Hedgcoth, unpublished observations). The difference in sequences between $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ is most likely only at position 37. This position in $tRNA_5^{Lys}$ is occupied by ms^2t^6A, whereas $tRNA_{5a}^{Lys}$ has the less modified t^6A. It appears that there is a precursor-product relationship between these two tRNAs.

$\underline{tRNA_6^{Lys}}$

$tRNA_6^{Lys}$ has been the subject of considerable research in our laboratory. This isoacceptor is associated with the mitochondria of some cells (38). It has been found only in virus-transformed cells and normal cells that have been stressed, e.g. by low serum levels. The source of $tRNA_6^{Lys}$ for sequencing was Balb/3T3 cells stressed by 1% calf serum in place of the usual 10% calf serum.

The structure revealed here shows an almost total deficiency in minor nucleotides. Of the 76 nucleotides in this tRNA, there are only 4 modified nucleotides: U*, t^6A, m^7G, and Ψ. This deficiency leads to the idea that $tRNA_6^{Lys}$ may be occurring from contamination of cell cultures with a simpler form of life, e.g. mycoplasma. While a contamination of this type is possible, it does not seem reasonable that a contamination not affecting cell growth could be present in a quantity to account for the amounts of

$tRNA_6^{Lys}$ that are observed. This isoacceptor has been found in amounts exceeding 20% of the total lysine tRNA. Several attempts to demonstrate mycoplasmal contamination of our cell lines have produced negative results. Furthermore, a mycoplasmal $tRNA^{Lys}$ chromatographs distinctly different from $tRNA_6^{Lys}$ (K. Thomas and C. Hedgcoth, unpublished observations).

Comparing known mammalian lysine tRNAs and all known lysine tRNAs, including eukaryotic and prokaryotic sources, there are a number of conserved sequences in $tRNA_6^{Lys}$ (Fig. 41). There are 24 nucleotides in eukaryotic tRNAs that are highly conserved (73). $tRNA_6^{Lys}$ has all except 4 of these nucleotides, but it has all of the 100% conserved nucleotides.

An unusual base pairing, G-G, is found in the acceptor stem. This is the only observation of such a pairing in an acceptor stem.

Special roles are expected to be attributed to U* and t^6A of the anticodon loop. U* may serve to restrict wobble; thus providing an explanation for the strong preference of $tRNA_6^{Lys}$ for the codon AAA (33). Nishimura has suggested that the presence of t^6A may assist in stabilizing hydrogen bonding between the U of the anticodon and the A of the first position of the codon (74). The presence of t^6A in position 37 is usually associated with a codon beginning with A. Randerath et al. (70) suggested that a concerted effort of U* and t^6A insure the correct orientation of the codon-anticodon interaction.

The GUΨC sequence in the TΨC stem and loop is undermodified as compared with the other lysine isoacceptors and the majority of eukaryotic tRNAs that have been sequenced. This region of tRNAs has been implicated in interactions between tRNA and the ribosome. S. epidermidis has a glycine isoacceptor that is involved in peptidoglycan biosynthesis (1, 2). The GTΨC

region of the glycine tRNA actually has GUGC. This alteration and perhaps other portions of the molecule prevent the functioning of this isoacceptor in protein synthesis. It is not known what effect undermodification of the normal tetranucleotide causes, but it is interesting to note the finding of Bjercke and Hedgcoth that $tRNA_6^{Lys}$ participates only about 25% as well as the other lysine isoacceptors in protein synthesis *in vitro* (33). Although functional aspects of the GUΨC sequence occurring in $tRNA_6^{Lys}$ can not be addressed further, it should be noted that a number of instances of eukaryotic tRNAs containing the sequence GUΨC have been reported: some of the $tRNA_4^{Lys}$ of SV40-transformed mouse cells (37); a *Drosophila* (75) and sheep liver (76) histidine tRNA; a wheat germ glycine tRNA (75); a mammalian phenylalanine tRNA (75); human placental (75), rat liver (77), and rat hepatoma (77) valine tRNAs. In addition, proline and tryptophan tRNAs from eukaryotic sources have the sequence GΨΨC (75).

Randerath *et al.* (70) proposed that modifying enzymes probably recognize secondary and tertiary structures rather than specific nucleotide sequences when selecting sites for modification except in the anticodon region. The nucleotide sequence is thought to be the determining factor in the modifications found in the anticodon region.

A recent observation has shown a profound effect of tertiary structure on the function of tRNA. Cordell *et al.* (78) showed that avian cellular $tRNA^{Trp}$ and the $tRNA^{Trp}$ that serves as a primer for avian myloblastosis virus reverse transcriptase have identical sequences. Reverse transcriptase binds the cellular form only 30% as well as the form isolated from virions. They suggested that the difference results from two conformations of this tRNA. In view of the ideas offered by Randerath *et al.* (70) on the

specificity of modifying enzymes, the questions perceived are: Do modifying enzymes function as well on both different conformational forms, or would the two forms be synthesized and modified in an identical fashion, and would the conformational change then occur as a postmodification event?

With these questions in mind, other questions arise concerning the undermodification of $tRNA_6^{Lys}$. Does the tertiary structure of $tRNA_6^{Lys}$ differ greatly from the tertiary structure of the remaining members of the lysine family? Is this a possible explanation for the undermodification of $tRNA_6^{Lys}$?

One residue of m^5C and one of D per molecule were found in the results of the total nucleoside composition. These nucleosides may have been contaminants because the sample of $tRNA_6^{Lys}$ used had not been through the final step of purification by electrophoresis on a polyacrylamide gel.

General comments regarding the lysine tRNA family

In each of the lysine isoacceptors studied, nucleotide 6 is m^2G except for $tRNA_6^{Lys}$. This modification has been found in two other tRNAs from abnormal cells. Piper (55) found this modification in a methionine tRNA from mouse myeloma cells, and Randerath *et al.* (58) reported it in a leucine tRNA from Morris hepatoma 5123D. Similar findings for some tRNAs from normal tissue were reported for a mammalian methionine tRNA (55) and for a histidine tRNA from sheep liver (76). Gupta *et al.* reported m^2G as nucleotide 6 in two glycine tRNAs from human placenta (75). Proline tRNAs from mouse and chicken tissues also have m^2G in position 6 (75). A tryptophan tRNA from bovine liver has a variation on this pattern with m^2G in position 7 of the acceptor stem (75). Other modifications of the acceptor stem are becoming increasingly common as additional sequences are obtained; 5-methylcytidine, pseudouridine, and 2'-O-methyl derivatives of

both uridine and cytidine have been reported in eukaryotic tRNAs (75, 76). The m^2G modification seems to be specific to eukaryotic tissues. A function has not been proposed for this minor nucleoside, whereas the methylation of guanosine in position 10 may increase the rate of aminoacylation (79).

Because $tRNA_{5a}^{Lys}$ in mouse liver and $tRNA_{6}^{Lys}$ in some transformed cells are associated with mitochondria, it was thought that these tRNAs may be encoded in mitochondrial DNA. Recently published data preclude this possibility. The sequence of the mouse L cell mitochondrial DNA has been reported (80). The DNA sequence presented in this paper is not complementary to either $tRNA_{5a}^{Lys}$ or $tRNA_{6}^{Lys}$. The $tRNA^{Lys}$ that would be transcribed from this sequence would be high in A-U content. This is a common feature of mitochondrial tRNAs. Randerath et al. (70) recently sequenced the mitochondrial $tRNA^{Lys}$ from rat liver, and it is very closely related to the lysine tRNA sequence encoded in mitochondrial DNA from mouse L cells. The two lysine tRNAs associated with mitochondria in studies from this laboratory are both 76 nucleotides in length, which is 10 nucleotides longer than the normal mitochondrial tRNA, and have a higher G-C content than do mitochondrial tRNAs.

In another recent publication, Nass (81) reported that the mitochondrial DNA is not perturbed by transforming viruses. This finding eliminates from serious consideration the idea that a virus somehow introduces a new tRNA gene into mitochondrial DNA.

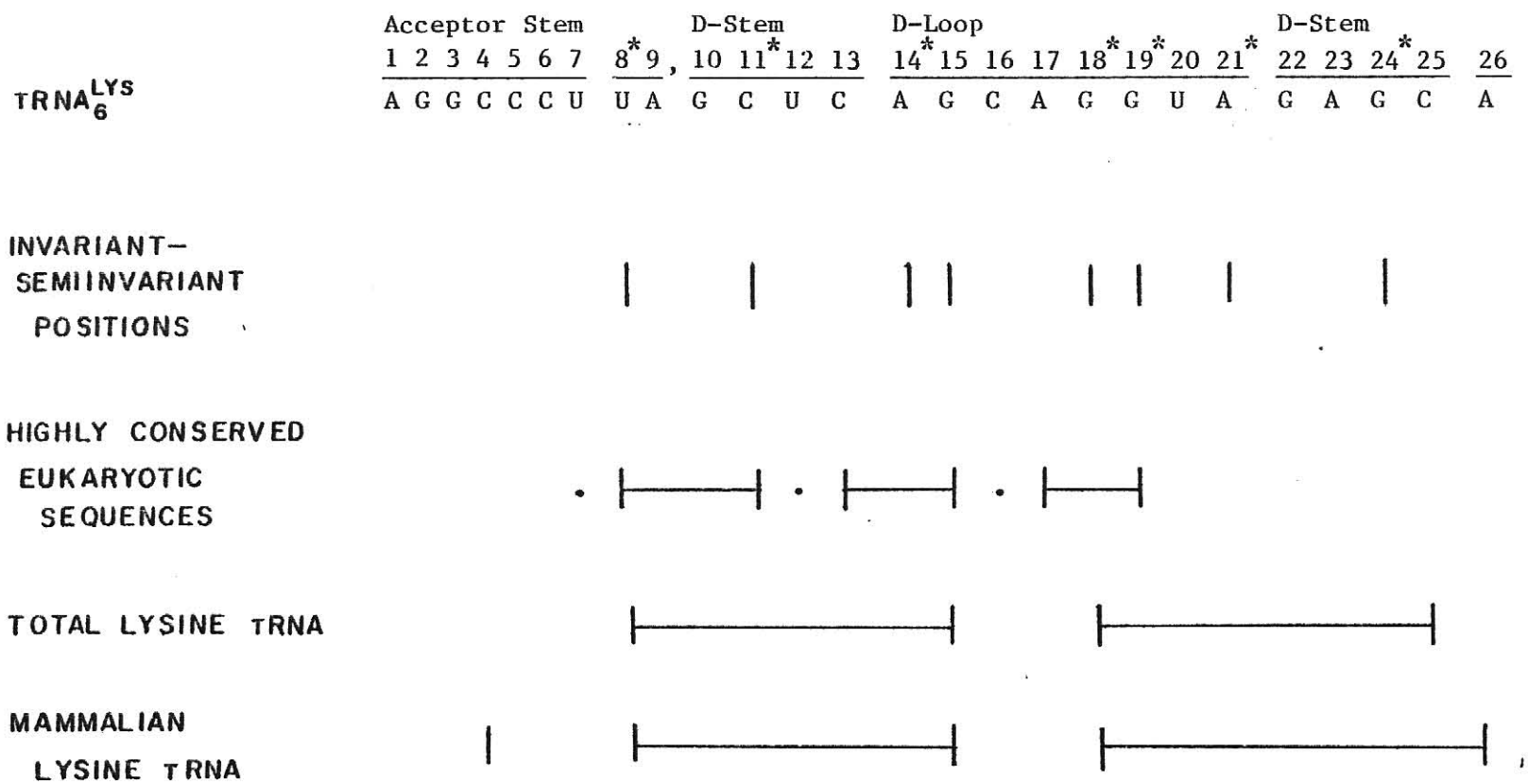
With these recent data in mind, I conclude that the mitochondrially associated tRNAs that we have studied are encoded in nuclear DNA.

The interrelationships of the lysine tRNA family have become quite complex and are beyond the scope of this thesis. Nishimura (82) has

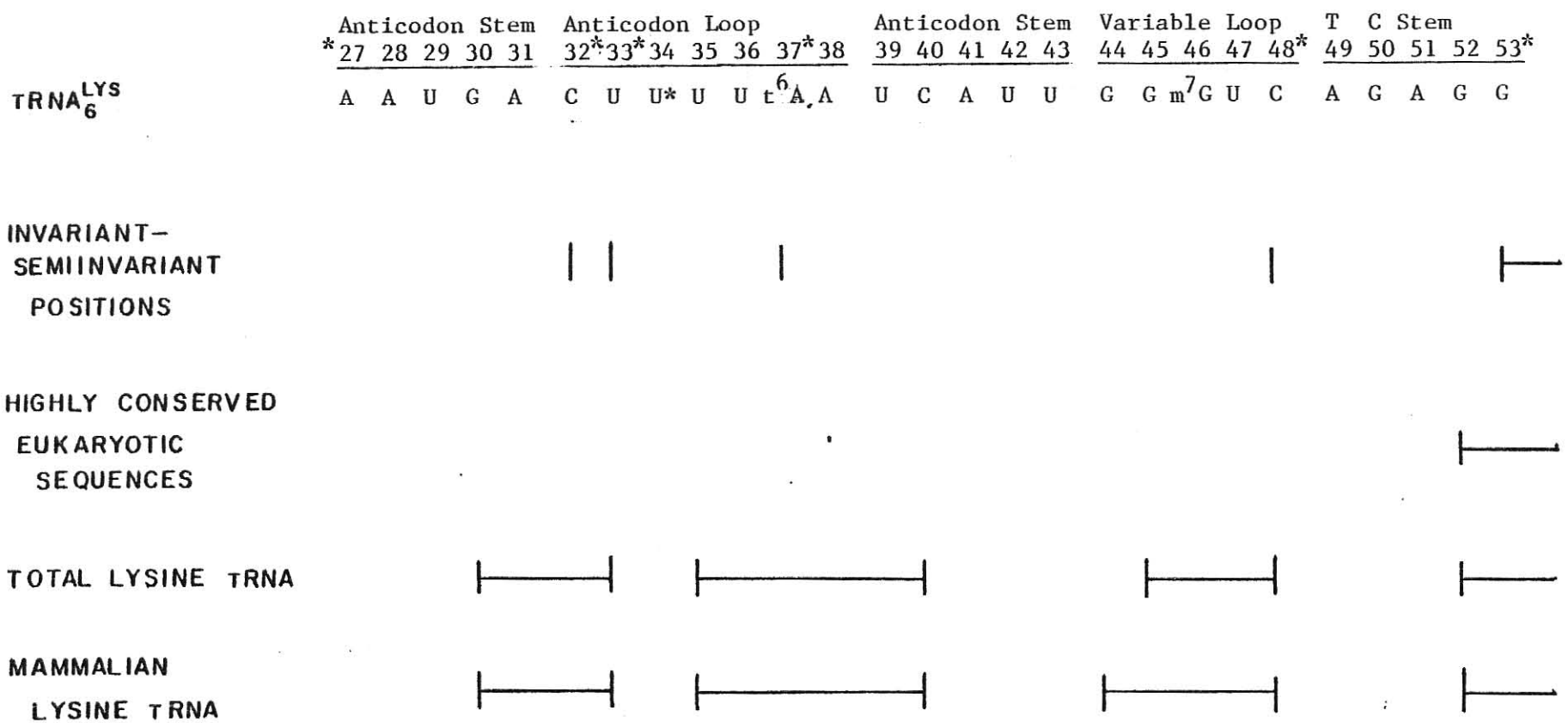
compiled a list of transformed tissue types and corresponding changes in isoaccepting tRNA profiles. The list indicates that there may be a number of systems rivaling the lysine family in its interrelationships. As more of these families are studied and analyzed, our knowledge and understanding of tRNA and its possible role in the maintenance of the transformed state will increase. The detailed study of the structures of tRNAs in the transformed state of cells is relatively new in the area of cancer research and should be a promising field for years to come.

Fig. 41. Conserved sequences in $tRNA_6^{Lys}$. This figure summarizes the sequences that are conserved in $tRNA_6^{Lys}$ as compared to invarient-semiinvarient positions found in tRNA (75), highly conserved sequences found in eukaryotic cells (73), the total lysine tRNA sequences known (75), and mammalian lysine tRNA sequences known (75). The nucleotides of $tRNA_6^{Lys}$ that are in agreement are marked with a line (bar). The nucleotides that are in disagreement are marked with a dot. $tRNA_6^{Lys}$ is totally conserved in reference to the invarient-semiinvarient positions. The invarient and semiinvarient positions found in tRNA are marked with an asterisk.

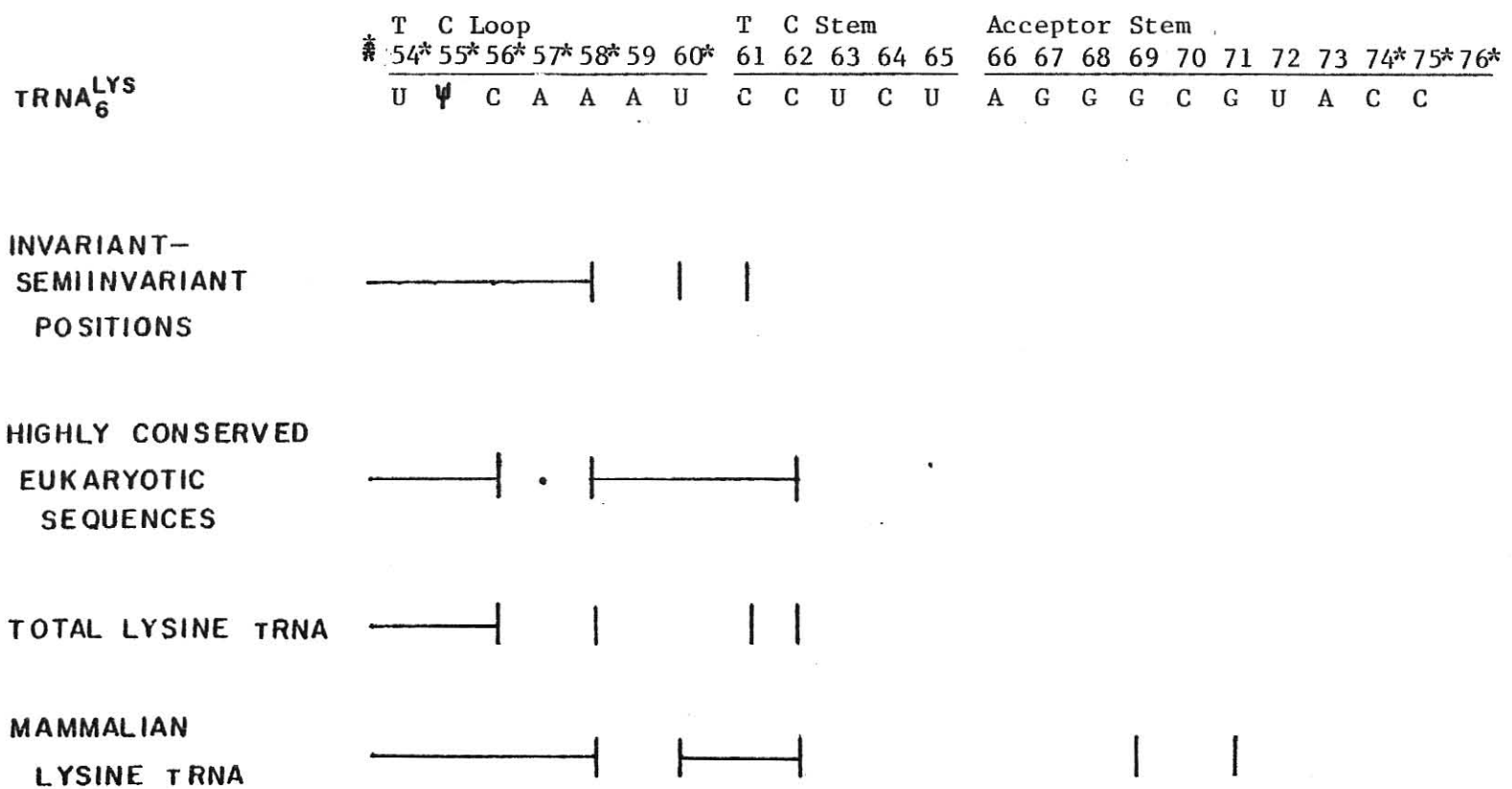
CONSERVED SEQUENCES IN $tRNA_6^{LYS}$



CONSERVED SEQUENCES IN $tRNA_6^{LYS}$



CONSERVED SEQUENCES IN $tRNA_6^{LYS}$



FOOTNOTES

[1]BD-cellulose, benzoylated DEAE-cellulose; RPC-5, reverse phase chromatography system number 5; RNase, ribonuclease; [5'-^{32}P]pCp, [5'-^{32}P]-cytidine 5', 3'-diphosphate; Um, 2'-O-methyluridine; Tm, 2'-O-methylribothymidine; t^6A, N^6-(N-threonylcarbonyl)adenosine; ms^2t^6A, 2-methylthio-N^6-(N-threonylcarbonyl)adenosine; mcm^5s^2U, 2-thio-5-methylcarboxylmethyluridine; pN, nucleoside 5'-monophosphate; pNp, nucleoside 3', 5'-diphosphate; SDS, sodium dodecylsulfate; TLC, thin layer chromatography; UV, ultraviolet.

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THE NUCLEOTIDE SEQUENCES OF FIVE LYSINE tRNAS FROM MURINE LYMPHOMA AND BALB/3T3

by

KIRK J. HAYENGA

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Graduate Biochemistry Group
Department of Biochemistry

KANSAS STATE UNIVERSITY
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The lysine tRNA family from mammalian cells can be separated into species 2, 4, 5, 5a, and 6 by RPC-5 chromatography. Isoacceptors $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$ are the major species of the lysine family. $tRNA_4^{Lys}$ is prevalent in dividing cells or cells capable of dividing. $tRNA_6^{Lys}$ has been found to be closely associated with the mitochondria in the transformed state, whereas $tRNA_{5a}^{Lys}$ has been found closely associated with mitochondria from mouse liver. Sequences of $tRNA_2^{Lys}$, $tRNA_4^{Lys}$ and $tRNA_5^{Lys}$ were determined by other researchers: $tRNA_2^{Lys}$ and $tRNA_5^{Lys}$ from rabbit liver and $tRNA_4^{Lys}$ from SV-40 transformed mouse fibroblasts. Isoacceptors $tRNA_{5a}^{Lys}$ and $tRNA_6^{Lys}$ have not been sequenced previously. There are literature reports of changes in the chromatographic profiles of tRNA families when comparing the tRNAs from a transformed cell line and a normal cell line. We sequenced isoacceptors 2, 4, 5 and 5a from cultured murine lymphoma cells (L5178Y) and isoacceptor 6 from stressed Balb/3T3 cells in culture. The Balb/3T3 cells were stressed by reducing the serum level of the medium from 10% to 1%.

The tRNAs were isolated from cultured cells by phenol extraction. Purification of the isoacceptors was accomplished by chromatography on RPC-5, derivatization to form naphthoxyacetyl derivatives and chromatography on benzoylated DEAE-cellulose. Each purified isoacceptor was electrophoresed on a one-dimensional or a two-dimensional polyacrylamide gel system to yield a tRNA purified for sequencing.

The total nucleoside composition was determined by a post-labeling method. Major sequence data were determined by either chemical sequencing or enzymatic sequencing. Additional information was achieved through random formamide hydrolysis, 5'-labeling with ^{32}P of the nested set of fragments, resolving the fragments using polyacrylamide gel electrophoresis

and identification of the 5'-labeled nucleotide. Identification of the 5'-labeled nucleotide was accomplished by freeing the nucleotide by enzymatic hydrolysis and chromatographing the enzymatic digest on polyethyleneimine cellulose thin layer plates. Further clarification was accomplished by two-dimensional thin layer chromatography.

The results indicate that $tRNA_2^{Lys}$, $tRNA_4^{Lys}$ and $tRNA_5^{Lys}$ closely resemble the structures previously reported for each isoacceptor. All three isoacceptors contain an m^2G in position 6 that was not found in the literature report.

The structures of $tRNA_2^{Lys}$ and $tRNA_4^{Lys}$ from lymphoma differ from each other by two nucleotides. Position 20 of $tRNA_2^{Lys}$ is a dihydrouridine, whereas $tRNA_4^{Lys}$ has an unmodified uridine in the same position. $tRNA_2^{Lys}$ has t^6A in position 37. This position in $tRNA_4^{Lys}$ is occupied by a modified nucleotide of unknown structure, which may be a hypermodified t^6A.

The sequences of $tRNA_5^{Lys}$ and $tRNA_{5a}^{Lys}$ are identical with the exception of position 37. This position in $tRNA_5^{Lys}$ is occupied by ms^2t^6A whereas $tRNA_{5a}^{Lys}$ contains the less modified t^6A.

The structure of $tRNA_6^{Lys}$ does not resemble that of the other lysine tRNAs presented in the thesis. This structure contains only five modified nucleotides U*, U**, t^6A, m^7G, and ψ.

The nucleotide sequence of mouse mitochondrial DNA has recently been reported. In conjunction with this and the sequence of a lysine mitochondrial tRNA from rat liver, it can be stated that $tRNA_{5a}^{Lys}$ and $tRNA_6^{Lys}$ are not encoded in the mitochondrial genome.