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Progress Report

During the past three years beginning October 1, 1977, we undertook several lines of investigation on our research program supported by the Department of Energy, Contract No. EY-76-S-02-3083. These included: 1) Restriction endonuclease studies of Tetrahymena mtDNA, 2) Biosynthesis of mitochondrial tRNA, 3) Structure and synthesis of tRNA charging enzymes, and 4) other pertinent research results.

This report describes our research accomplishments and activities during the past three year period.

1) mtDNA mapping

At the end of 1977 we accumulated substantial data on restriction fragments of Tetrahymena mtDNA, particularly strain ST. The fragment analysis data with EcoRI, Hpa II, Hind II, Hind III, and a tentative map of ST mtDNA were presented in my research proposal submitted to the U.S. Energy Research and Development. While we continued to work on this project, Goldbach and his colleagues at P. Borst laboratories in Amsterdam carried out extensive mapping analyses and other structural studies on mtDNA of ST strain which P. Borst originally acquired from me. The data of Goldbach et al (1977, 1978), however, contradicted my tentative data in details. Particularly disturbing was the fact that EcoRI fragments to which 21S and 14S rRNA hybridized showed a great deal of discrepancy. Further studies I have undertaken since, however, have confirmed Goldbach's data. My hybridization data differed from theirs because my rRNAs, 21S and 14S, were collected from sucrose gradient centrifugation of in vivo labeled RNA, whereas Goldbach et al (1978) collected their rRNAs from agarose gels, which were made radio-active in vitro. Although 21S and 14S rRNA in our preparations were purified through two rounds of sucrose gradient centrifugation, they appear to have been contaminated with other RNAs. The 21S rRNA isolated through sucrose gradient hybridized mainly with EcoRI fragment-2 most intensively; this was previously used for our mapping. On the other hand, in vitro labeled, agarose-gel isolated 21S RNA hybridized with fragments 1 and 3, instead of fragment 2. These results are now more carefully reviewed in an attempt to resolve the nature of 21S rRNA prepared through sucrose gradients.

Several strains of Tetrahymena which we tested were obtained from the following sources: Dr. M.A. Gorovsky at Rochester provided T. thermophila and GL strain (originally obtained from I.L. Cameron), Dr. H. Sato at Nagoya University provided a GL strain (originally received from Dr. G.W. Kidder), and Dr. H.J. Eichel provided W and E strains. Of these strains, Dr. Sato's GL strain contained mtDNA which upon EcoRI treatment produced essentially the identical fragment pattern as that of ST mtDNA, except for the size of fragment 3 in GL, which is noticeably smaller than that of ST. We have also hybridized ST rRNAs and found that they hybridized with fragments 1 and 3 as with ST DNA fragments. We are examining sucrose gradient isolated tRNA (21S) to see if it will produce the same hybridization as with ST DNA.

Other restriction enzymes such as Bam I, Hae II, Hae III, and EcoRII were also tested with ST DNA. In our analysis, Hae II produced 2 pieces of 11.5×10^6 , one piece each of 2.9, 2.4 and 2.1×10^6 MW DNA fragments. Hpa II produced 1 x 6.5, 1 x 5.6, 2 x 4.3, 2-3 x 1.7, 1 x 0.67, 1 x 0.43, 1 x 0.31×10^6 ; Hind III, one piece each of 7.3, 4.8, 3.2, 2.4, 2.1, 1.2, 0.71, and 0.43×10^6 MW fragments. Bam I from Miles and Hae III from BRL produced no restriction fragment.

2) Biosynthesis of tRNA

To study mitochondrial tRNA and its biogenesis in detail as proposed earlier, it becomes apparent that more sensitive separation methods are needed than MAK and RPC-5 column chromatography and one-dimensional polyacrylamide gel electrophoresis. Two-dimensional urea-polyacrylamide gel electrophoresis developed by Fradin et al (1975) is obviously better suited for our purposes.

With some modification of Fradin's method we achieved fair separation of mitochondrial and cytoplasmic tRNAs isolated from Tetrahymena. The primary purpose of these experiments were: (1) to compare mitochondrial and cytoplasmic tRNA species by 2D polyacrylamide gels, (2) to reexamine our previous conclusion that Tetrahymena mitochondrial tRNAs consist of nuclear-DNA-coded tRNAs, as well as mtDNA-coded tRNAs, (3) to see if we could detect any precursor tRNA molecules from mitochondrial tRNA, and (4) to determine if 5S rRNA does indeed exist in Tetrahymena mitochondria.

I briefly summarized some of my 2D-urea-polyacrylamide gel data in an earlier Progress Report (1979). I am doing this again and include new data on mitochondrial tRNA in this Report.

Tetrahymena mitochondrial tRNA was claimed to be composed of what we called native and imported tRNAs (Chiu, Chiu and Suyama, 1975). 2D urea-polyacrylamide gel electrophoresis of mitochondrial tRNA produced about 30 tRNA spots in the gels, and whole-cell tRNA produced about 45 spots. Our earlier gel system consisted of 40 cm 1st D of 4M urea-10% polyacrylamide gel and 2nd D slab gel of 4M urea-20% polyacrylamide. Comparisons of mitochondrial and whole-cell tRNAs developed in 2D gels, showed that most of the tRNA spots between them appeared to be identical in their positions in the gels. However there were several mitochondrial tRNA species which were unique to mitochondrial tRNA and absent in whole-cell tRNA. A mixing experiment of 32 P-labeled mitochondrial tRNA and cold whole-cell tRNA produced the same result. It should be mentioned that major and minor cytoplasmic tRNA isoacceptors were not necessarily represented proportionately in mitochondrial tRNA in comparison of the gels. Namely, a major cytoplasmic tRNA appears as a minor tRNA in mitochondria, or vice versa. However, their relative concentrations appear to be constant in different preparations of mitochondrial tRNA. Even early and late exponential cells produce essentially similar mitochondrial tRNA patterns in relatively constant proportions.

In order to evaluate the transcriptional origin of mtRNA, ³²P-labeled mitochondrial tRNA was prepared from in vivo labeled cells hybridized to mtDNA. tRNAs were stripped from the hybrid and were run in 2D gels. An autoradiogram developed only 6 radioactive spots in contrast to a total of up to 30 mitochondrial tRNA spots. We have since improved our gel electrophoresis by adding an additional 15% polyacrylamide gel layer on top of 20% acrylamide gel. This disc 2D gel produced better overall separation of some tRNAs, resulting in about 36 spots. Hybridized tRNAs in this gel system produced 10 radioactive spots in the gels.

Amino acid accepting capacities of some of these tRNAs were determined. We have now identified 2 methionyl, 1 phenylalanyl, 1 leucyl, 1 tyrosyl, and 1 tryptophanyl tRNAs as being hybridizable (or native) tRNAs. However, the trp and tyr tRNA and one unidentified tRNA (perhaps leucine tRNA) migrate in a cluster, which makes it difficult to identify them convincingly. Three additional autoradiographic spots have not been identified. One of them cannot be acylated with the 12 amino acids tested so far. This one plus two others do not reproducibly appear; they may represent partially degraded tRNA.

Many have argued that Tetrahymena mitochondrial tRNAs are contaminated with cytosol tRNAs, despite the fact that I have presented much strong circumstantial evidence against this possibility. In the past two years I have attempted to obtain more direct evidence which has been made possible due to the development of 2D gel electrophoresis. I present here three such pieces of evidence.

(1) When cells were homogenized for isolation of mitochondria, ³H-labeled Tetrahymena whole-cell tRNA was added. Mitochondrial tRNA isolated from this cellular homogenate did not contain any traces of radioactivity. The 2-D gel pattern of the isolated tRNA is, of course, identical to the usual one.

(2) When the cell homogenate was treated with RNase A (25 µg/ml) in the cold for 20 min, the mitochondrial tRNA which was subsequently isolated developed the identical 2D gel pattern,

(3) I have isolated 80S mitochondrial ribosomes from the sucrose gradient as described before (Chi and Suyama, 1970). Incidentally, the contamination level of the cytoplasmic 80S ribosome was estimated to be less than 0.2% by the CsCl gradient centrifugation analysis of the isolated ribosomes. tRNA was then purified from the 80S mitochondrial ribosomes, and analyzed in 2D gel. I found that this tRNA displayed the same basic pattern as nonbound tRNA. I used nonradioactive as well as ³²P-labeled cells from which mitochondrial 80S tRNAs were isolated. Both produced the same results. The steps used to isolate these mitochondrial were quite stringent; I believe the chances of having contamination of cytosolic tRNA to be extremely low. I might add that the proportions of all tRNAs are relatively constant, but the ribosome bound tRNA had slightly different proportions from those of the nonbound tRNAs.

This last result further indicates that all the mitochondrial tRNAs are involved in intramitochondrial translation.

A few words should be added concerning the state of precursor tRNAs and 5S rRNA in mitochondria. The 2D gel runs containing mitochondrial tRNA obtained from ^{32}P -grown cells (a semi-steady state) showed no significant radioactive spot other than tRNA. We found however, a faint spot having the same mobility as the cytoplasmic 5S RNA in 1st D gel, but having a greater mobility in the 2nd D gel. This species hybridizes with mtDNA; the RNA stripped from the hybrid shows the same mobility as the original one. I am currently investigating this RNA further.

(3) Structure and Synthesis of tRNA charging enzymes.

We compared the mitochondrial and cytoplasmic tRNA synthetases more specifically than we had reported previously (Suyama and Hamada, 1978). The mitochondrial and cytoplasmic valyl tRNA synthetases could not be differentiated by many physical and chemical parameters used before such as DEAE-cellulose, hydroxylapatite and phosphocellulose column chromatography, K_m 's for ATP, tRNA and valine, acylation reaction temperature, as well as sensitivity to heat inactivation, and substrate affinity toward E. coli, yeast and rat liver tRNAs (Suyama and Hamada, 1978). In order to characterize these enzymes immunologically, I have attempted to purify the cytoplasmic enzyme for antiserum preparation. Ammonium sulfate fractionations, sucrose gradient centrifugation with tRNA, isoelectric focusing agarose and Cello-Gels were combined. However, the resulting protein was not yet pure and produced three to four major bands and several minor bands. More time is needed to accomplish the desired purification.

The regulation of tRNA synthetases in response to drugs such as chloramphenicol and cycloheximide was examined. The enzymes examined were: valyl, tyrosyl and phenylalanyl RNA synthetases. The mitochondrial and cytoplasmic extracts obtained from Tetrahymena cells with or without drug treatment for various periods of time were analyzed for these synthetase activities by sucrose gradient centrifugation. In general, we have found that the mitochondrial enzymes for tyrosyl and phenylalanyl tRNAs (native tRNAs) accumulated in drug-treated cells, whereas the valyl tRNA (imported) synthetase showed very little change by drug treatment. We have prepared a manuscript describing this aspect of research which will be submitted for publication.

(4) We have not carried out experiments directly related to the mitochondrial ribosomal proteins. We have, however, acquired techniques for separating 55S subunits by agarose isoelectric focusing. The reproducibility of the method is not good at this time, and more improvement is necessary.

We have worked on several other areas of research related to the proposed research projects.

Edward Fliss, who has just completed his Ph.D. dissertation, succeeded in characterizing whole-cell messenger RNA activity in various Tetrahymena cell cultures (Fliss and Suyama, 1979). Isolated poly A⁺ RNAs were assayed for high molecular weight protein production in the in vitro wheat germ translation system. This area of study was needed to advance our system to test the transport of extramitochondrially translated proteins to the mitochondria.

Edward Fliss also helped to establish O'Farrell's two-dimensional gel electrophoresis of proteins. He established the separation of Tetrahymena axoneme tubulins by this system; the results of this have been submitted for publication to J. Protozool. We also made several gel electrophoresis runs of bulk mitochondrial proteins by the gel system. We found a number of well defined protein spots in the area of gel with pH higher than 7 and MW below 50,000 daltons. Interestingly, most proteins appeared as a series of isoelectric variants. It would be of interest to examine whether these variable forms result from post-translational modifications. Nevertheless, we are in a position to analyze mitochondrial translation products as well as transported proteins in the well defined 2D gel.

Finally I have obtained technical collaboration from Dr. Narayan Avadhani of this University in analyzing DCCD binding protein of the ATPase of Tetrahymena mitochondria. ³H amino acid incorporation into the protein by isolated mitochondria apparently did not occur. This work has to be further extended to obtain more conclusive evidence, but a possibility exists that Tetrahymena mitochondrial DCCD binding protein is translated extramitochondrially as in Neurospora.

Publications and Other Activities Resulting from the Department of Energy
Contract EY-76-5-03-3083:

Suyama, Y. and J. Hamada (1978). The mitochondrial and cytoplasmic tRNA synthetases are indistinguishable. Arch. Biochem. Biophys. 191, 437-443.

Fliss, E. and Y. Suyama (1979). Tetrahymena tubulins and in vitro translation of Tetrahymena RNA. J. Protozool. 26, 505-509.

Fliss, E. and Y. Suyama (1980). Two dimensional electrophoretic analysis of isolated cortical structures. Submitted to J. Protozool.

Hamada, J. and Y. Suyama (1980). Differential regulation of the mitochondrial and cytoplasmic tRNA synthetases for imported and native tRNAs. Manuscript in Preparation.

Suyama, Y. (1980). Two dimensional polyacrylamide gel electrophoresis of Tetrahymena mitochondrial tRNA. Manuscript in Preparation.

Ph.D. Students trained:

Jin Hamada (1978), Ph.D. Dissertation Title: Native and Imported tRNAs and their Synthetases in Mitochondria of Tetrahymena pyriformis. Kyoto University, Kyoto, Japan.

Edward Fliss (1980), Ph.D. Dissertation Title: Tubulin Synthesis in Tetrahymena and Analysis of Isolated Cortical Structures, University of Pennsylvania.