Summary of Symposium on Transfer RNA and Transfer RNA Modification in Differentiation and Neoplasia

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It is over a decade since the discovery of tRNA and 5 years since the elucidation of the first primary sequence. The complete primary structures of approximately 19 tRNA's are now known, and without exception the clover leaf configuration has provided a representation for the secondary structure which is theoretically satisfying. The tertiary structure is still, however, a hazy subject; we await results of X-ray diffraction of crystalline tRNA's, with the use of isomorphous replacements, for clarification and possible decision among the several structures proposed.

tRNA has long been known to be rich in minor base modifications (6, 20), of which the most common is methylation, and this occurs on all 4 major bases. Data presented at this symposium by Stulberg and Shugart (72) support the logical inference that these base modifications change the tertiary structure of the tRNA's and modulate its several functions. The data of Carbon and Squires (15), using tRNA$_{18S}$ and the glycine-activating enzyme, and of Gefter and Bikoff (29), using the tRNA$_{Tyr}$ system of the Cambridge group (1, 2, 30), implicate the anticodon in amino acid recognition as well as in the established interaction with the codon triplet of the mRNA. These studies add substance to earlier data pointing in this direction, including the suggestion from our laboratory (13) that the 2nd position in the anticodon may be used as part of the aminoacyl synthetase recognition information.

The evidence of Shugart and Stulberg (67), using borohydride reduction of intact tRNA$^{Phe}_{e}$ points to the dihydrouracil loop as being essential for aminoacyl synthetase recognition. In this connection, it is important to note, as these investigators did, that this effect may be either direct or indirect. The 2-dimensional projection of tRNA in the clover leaf configuration fails to show the rotational translation which occurs in each of the double-stranded stem regions. In general, these 4 double-stranded stems (of the major loops plus the $-CCA$ end of the molecule) are quite free of modifications of the major bases. In fact, there are no minor bases in the double-stranded heptanucleotide of the $-CCA$ region. Chemically or mutationally induced minor base changes in the double-stranded regions, by impairing hydrogen bonding or base stacking, would be likely to produce a topological change in the relationship of the loops of the molecule toward each other or toward the $-CCA$ aminoacyl acceptor site. Thus there is no assurance that alteration of a single base (outside of the anticodon triplet) has a direct effect on its aminoacyl acceptor or ribosomal association functions. There is now no evidence to rule out the suggestion that the aminoacyl synthetase recognition site may be constituted from more than 1 loop area, with tertiary folding of the tRNA molecule bringing the active site into being (87).

The data of Shugart et al. (66), Stulberg and Shugart (72), and Peterkofsky et al. (57) help to clarify the role of methylation of tRNA in aminoacyl synthetase recognition. Using enzyme-saturating concentrations of tRNA$^{Phe}_{e}$ and initial aminoacylation rates, Stulberg reports that undermethylated tRNA cannot be aminoacylated as well as normally methylated tRNA can be, a conclusion with which the work of Peterkofsky (using tRNA$^{Met}_{e}$) agrees. Peterkofsky also finds that, while normal tRNA$^{Leu}_{e}$ is acylated by both Escherichia coli and yeast aminoacyl-tRNA ligases, methyl-deficient tRNA$^{Leu}_{e}$ is acylated only by the E. coli enzyme. Furthermore, while normal tRNA$^{Leu}_{e}$ recognizes both poly UC and poly UG, methyl-deficient tRNA$^{Leu}_{e}$ recognizes only poly UC. Finally, he reports that undermethylation results in decreased ability of tRNA$^{Leu}_{e}$ to bind E. coli ribosomes. Stulberg and Shugart (72) report that overall protein synthesis, as reflected by poly U-stimulated phenylalanine incorporation, is also decreased in rate in the undermethylated species. Studies on physical parameters of tRNA by the same investigators show a change in circular dichroic and optical rotatory dispersion patterns and in melting profiles of undermethylated tRNA$^{Phe}_{e}$ as compared with the fully methylated tRNA$^{Phe}_{e}$.

The use of bacterial mutants with defects in tRNA-methylating enzymes would appear to be helpful in pinpointing the role of methyl groups in the functioning of tRNA. Björk (8, 9), now working with Neidhardt (56) on growth control mechanisms involving tRNA, has in fact isolated an E. coli mutant lacking $5$-methyluridine in its tRNA. Only small but significant decreases from normal were observed in the rate of growth of this mutant and in the rate of overall protein synthesis and in translation efficiency. It still remains a possibility that the conditions used for these experiments were not such as to make the rate of the reaction being scrutinized the limiting one. Nevertheless, at present Björk's findings fail to support those mentioned previously on the role of methylation in the biological functioning of tRNA.

The work of Carbon and of Gefter in this symposium and elsewhere (14–16, 29, 30, 41) points up the potential power

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of the use of mutational changes in tRNA in the study of these structure-function relationships. Carbon and Squires (15), in particular, emphasize the greater specificity and freedom from confusing side reactions of mutational alteration of tRNA as compared with chemically induced changes. It is clear that this approach, i.e., genetic engineering, holds promise for solution of the recognition reaction problem.

Hall et al. (35) mentioned that from their work, that of Skoog and the Wisconsin-Illinois group, and that of others (cf. Ref. 68), it appears that for codons with uracil in the 1st position (i.e., UNN codons, where N refers to any 1 of the 4 ribonucleotide bases) that an isopentenyladenyl residue or a modification thereof is in the position adjacent to the anticodon base which pairs with the uracil residue in position 1 of the codon triplet. Newer data from Dyson et al. (21) indicate that N-[purin-6-ylcarbamoyl]threonine is located in the residue adjacent to the anticodon base which pairs with an A situated in the first position of a codon triplet. Incidentally, N-[9-(β-D-ribofuranosyl)purine-6-ylcarbamoyl] glycine has also recently been isolated from enzymatic digests of unfractionated yeast tRNA (65). Both isopentenylenosine and modification of the carbamoylthreonine adenosine residues are powerful cytokinins. The work of Gefter and Bikoff (29) with tRNA mutants also indicates that the modification of the base adjacent to the anticodon in tRNA is related to the code words used. Removal of the modification impairs codon-anticodon interaction by reducing binding of the tRNA to ribosomes (30). Hall hinted that it is conceivable that there may also be particular base modifications adjacent to anticodons which pair with cytosine and guanine in the 1st codon positions, although there is presently no firm experimental support for this suggestion. There is, however, sufficient evidence from the above-mentioned data to point out that positioning of an amino acid in a growing peptide chain, with the high degree of fidelity which is characteristic of protein synthesis, may involve more than a triplet codon-anticodon interaction. This point of view was forecast in the early days of the coding era by Loffeld (49), who based his opinion on the energetics of the selectivity process. Fidelity of translation, as has been mentioned in this symposium by Borek (10), may be a function of tertiary structure of the tRNA molecule.

Little direct attention was paid at this symposium to the nature of the interaction between an aminoacyl-tRNA ligase and its cognate tRNA. It is surprising how little progress has been made toward elucidation of the molecular nature of the code which must exist between a few key side chains of the amino acids of the aminoacyl-tRNA synthetase and certain bases or modified bases of the cognate tRNA.

Tchou et al. (75) described their purification of tryptophan-tRNA synthetase from human leukemic blood. One pint of blood may provide an amount of starting material (around 200 g of lymphoblasts) sufficient for enzyme purification comparable to the scale used for E. coli. By means of column chromatography, a 240-fold purification was obtained, and another 10-fold purification was achieved by isoelectric focusing. The enzyme was partially characterized with respect to stability, kinetics, and specificity, and a molecular weight of the order of 90,000 was obtained. It was found to charge tRNA as well as a substrate. Whether it dissociates into subunits has not been determined.

**Alterations of tRNA during Differentiation**

At this point, the symposium moved from a biochemical characterization of tRNA's to a consideration of alterations of tRNA during differentiation. Separate data of Taylor [Holland et al. (42); Taylor et al. (73, 74)], Mittelman (53), and Yang (85, 86) support the conclusion that there are more isocodon tRNA species in embryonic forms of a tissue than in the adult tissue. Taylor [Taylor et al. (73, 74)] compared tRNA's from unfertilized sea urchin eggs with those from 24-hr blastulas and tRNA's from hepatomas. Using methylated albumin-Kieselguhr columns and reversed phase column chromatography, as introduced by Weiss and Kelmers (84), he found both quantitative and qualitative changes in specific tRNA elution patterns. He was led to consider that genetic redundancy and differential gene transcription may be involved, in explanation of the above results, and in agreement with a conclusion of Russell et al. (64). His experiments also suggested, in line with recent work of Burdon and Clason (12) and of Bernhardt and Darnell (7), and as presented here also by Gefter and Bikoff (29), the presence in his cellular RNA separations of RNA molecules which are precursors to tRNA.

Hatfield et al. (39) found differences in elution profiles (using Kelmets-type columns) of methionyl-, arginy1-, and seryl-tRNA's from bovine brain and liver. No differences in elution profiles of the phenylalaninyl-, lysyl-, and leucyl-tRNA's from brain, liver, and kidney nor in elution profiles of methionyl-, arginyl-, and seryl-tRNA's of liver and kidney were observed. Furthermore, codon responses of fractionated seryl-tRNA were reported. One species of seryl-tRNA responded specifically to the codon, UGA. These organ-specific differences in chromatographic elution pattern, and the codon response to UGA have no ready explanation. Great pains were taken to rule out or minimize possible artifactual differences that might be related to isolation procedures.

Mittelman (53, 55) extracted tRNA from spleens of patients with malignant lymphoma and leukemia and from nonneoplastic diseases. Fractionation of these tRNA's on benzoylated diethylaminoethyl cellulose columns (33) revealed the presence of 2 phenylalanine acceptor tRNA's in nonneoplastic spleens, while from the neoplastic spleens there were at least 3 phenylalanine acceptor tRNA peaks. When these tRNA's were charged with aminoacyl synthetase extracted from human embryo livers, many isococetupling tRNA subtypes could be observed. There was suggestive evidence that the number of aminoacyl synthetases and their activities might vary with the age of the embryonic tissue. Yang (85, 86) extracted tRNA from 2 mammalian tumor systems, L-m tumor cells (both in tissue culture and in the mouse), and Reuber hepatomas; and from 3 control tissues, regenerating, fetal, and adult normal livers. Using reversed phase chromatographic columns, he found that the elution patterns for the tRNA's from tumors were very different from those of the control tissues for the asparagyl-, histidyl-, tyrosyl-, and threonyl-tRNA's. Isoaccepting tRNA's of tumor tissues.
such as aspartyl- or tyrosyl-tRNA's could be divided into 2 sets. One tRNA similar to the normal, differentiated tissue and one tRNA not found in the differentiated tissue were also found in embryonic tissue (term fetus liver), although in lower quantity in the latter.

Results parallel to these have also been reported in differentiating plant tissues (18), and in sporulating bacteria (48). These various findings agree with the hypothesis (71) that differentiation and loss of synthetic capacities characteristic of cell growth are associated with loss of specific isoacceptor tRNA subspecies, with consequent codon restriction.

Alterations in tRNA and tRNA MethyInes in Neoplasia

Mandel [Hacker and Mandel (34); Mandel et al. (52)] found suggestive evidence of hypermethylation of tRNA in Marek's disease (the cell-associated herpes virus oncogenic disease) in infected chicken livers as compared with normal livers. He did find aberrant methylation in vitro with the neoplastic liver extracts as compared with the normal liver, with, in 5 methyl uracil, 7 methyl guanosine, and 3 methyl-cytosine. In vitro tRNA methylase activity was elevated 3-fold in the tumors.

The question was raised here, and in subsequent papers involving comparisons of methylases and methylating patterns in malignant and control normal tissues, as to what role the change in histological cell type distribution between the tumorous tissue and the tissue from which it was derived might play in the change in methylase and in methylation pattern. There was no easy answer to this question, which has of course troubled investigators in the cancer field for years, whenever a comparison is made between constituents of malignant and normal tissues. This point was also made in a study of enzymatic methylation of tRNA by Gantt (27, 28) as reported at this meeting. The necessity for careful histological controls in comparisons of methylase and minor base contents of tRNA's of a large number of human brain tumors as compared with normal tissues (6, 55). On the other hand, in some published reports (3, 43), base constituency data of tumors are in agreement with certain other published data on minor base groups in normal brain tissue. The question was raised by Weinstein (82) as to whether such differences might reflect, as mentioned previously, histochemical variability, as for example between the amount of gray matter versus white matter of the malignant as compared with the normal tissue. Here again, there seemed to be no ready answer to the query.

Randerath (61, 62) then reported details of a sensitive technique for identifying and determining quantitatively the minor base constituents of tRNA. This procedure depends on degradation of the tRNA enzymatically by nucleases and phosphomonoesterases to the mononucleoside level. Next, the mononucleosides are oxidized by periodate; then they are reduced to the triolcohols by tritiated borohydride of very high specific activity (cf. also, Refs. 47 and 59). These triolcohols of the mononucleosides are next separated by 2-dimensional thin-layer chromatography on cellulose. They are then located by fluorography, the latter procedure being enhanced (60) in sensitivity by addition of scintillator directly to the cellulose plate and by exposure to sensitive X-ray film at —80°. Using this procedure, Randerath found no differences in minor base contents between several tumors and normal tissues, thus introducing a disquieting note in the hitherto generally agreeable preceding reports. There was no ready reconciliation of these findings with those of Viale (79, 80) nor with certain other published data on minor base constituency of tRNA from tumors as compared with normal tissues (6, 55). On the other hand, in some published reports (3, 43), base constituency data of tumors are in agreement with the data of Randerath. There was also a conflict between the report of a high N'-methylguanosine content of hamster liver tRNA by Mittelman et al. (54) and the value obtained by Randerath (61).

In the presence of some of the uncertainties mentioned above, due in reality to the complexity of the biological system under study and to the difficulties of isolating minor bases, the reports of Kerr (45) and Kerr et al. (46) were both very interesting and an added source of worry to those studying methylases. At this meeting, we seemed to be in the position of one taking an automobile off the assembly line before completion and driving off while trying to attach a wheel. We are trying to explore the biochemical anatomy of the machinery of growth and at the same time to apply it to the problems of differentiation and neoplasia.

Kerr (45) found 2 methylase-inhibitory factors in the 100,000 X g, pH 5 supernatant fraction of rabbit liver. Together, these factors complexed with rabbit liver methylase and inhibited its action. One of these factors was a protein with a molecular weight of 100,000, and the other was a small peptide with a molecular weight of below 700. Fetal livers and certain tumors were found to lack the large-molecular-weight protein-inhibitory factor. It is thus possible that these 2 factors (cf., also Ref. 17) and a somewhat different methylase-inhibiting factor, which Wainfan (81) mentioned in her bacterial methylase studies, may play a role in the control mechanisms involved in differentiation.

There were several papers on chemical carcinogenesis. The presentation of Weinstein et al. (83) was a nice description of
molecular oncology, in pointing out a rather specific attachment of the carcinogen acetylamino-fluorene to position 8 of a guanine residue in tRNA. Model studies reinforced the impression that the attachment of the carcinogen to this position would interfere both with the hydrogen bonding and with the single chain base stacking of the affected base.

Magee (50, 51) studied the relation of chemical methylation to carcinogenesis and concluded that, while there is no clear indication of which bases of nucleic acids are critical for carcinogenesis, tRNA may be a crucial target.

In relationship to chemical carcinogenesis, Hancock (36–38) studied details of the methylation reaction itself, described an ammonium ion enhancement effect on methylase (cf. also, Refs. 44 and 63), and endeavored to define the conformational requirements for methylase activity. He also posed the rather bold question, suggested likewise by Weinstein et al. (83), of whether all carcinogenesis might be due to alterations of minor bases of transfer RNA. Weinstein had presented a diagram suggesting that there may be some normal feedback from tRNA to the genetic material. Such a possibility does not seem as remote as it might have appeared before the Temin-Baltimore (5, 76) enzyme became well recognized. Incidentally, since the term “retroscription” has been used in parlance to describe the action of this RNA-DNA polymerase, the colloquial name “reverscriptase” may be suggested for it.

It may be interesting to recall that we have recently had a defense of the Central Dogma presented nicely by Crick (19). According to the current formulation of the directions of flow of information in the living cell, protein is not synthesized when protein is used as a template, but recent work of the Lipmann (31, 32) and the Laland (24) groups shows that the peptide antibiotic, gramicidin S, containing 10 amino acids, can be synthesized on an enzyme surface without the intervention of an RNA template. This finding raises thoughts about the time in evolutionary molecular biology when tRNA may have been inserted. If one searched hard enough among primitive living species, there might be found not only precursor molecules of tRNA which are 20 residues or so longer than tRNA itself, but possibly simpler, shorter models of tRNA, sparse in minor base constituents. These perhaps would be incapable of the sophistication which tRNA’s now have in translating the message of the nucleotide to that of the protein.

Let us mention that Aelius Donatus said in the 4th century A.D., “Perish those who said our good things before we did.” The last speaker at this symposium is, contrariwise, indebted to the preceding speakers for helping to clarify the role of methyl groups in the functioning of tRNA. Finally, we are grateful to Dr. Borek for bringing this symposium together and for pointing his finger so early (4, 22, 23, 69, 70, 77) at the relationship of methylation of tRNA to problems of growth and neoplasia.

REFERENCES


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