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Alanine transfer RNA

Nobel Lecture, December, 12, 1968

Work on the alanine transfer RNA actually began in 1956 in James Bonner's laboratories at the California Institute of Technology. I was on sabbatical leave from the Geneva Experiment Station of Cornell University and was studying protein synthesis. Toward the end of my leave I carried out experiments designed to detect the acceptor of activated amino acids.

At that time it was already known from the work of Hoagland, Keller and Zamecnik[1], DeMoss, Genuth and Novelli[2], and Berg and Newtons that amino acids are activated enzymatically to give enzyme- bound amino acyl-adenylates (Enz-AA-AMP, Fig. 1). It seemed likely that these amino acyl-adenylates would react with something, indicated as <X> in Fig. 1, and one product of the reaction would be AMP (adenosine 5' -monophosphate), as formulated in the second equation in Fig. 1. It seemed quite possible that such

$$\text{Enz} + \text{AA} + \text{ATP} \rightleftarrows \text{Enz-AA-AMP} + \text{pyrophospha}$$
$$\text{Enz-AA-AMP} + \langle X \rangle \rightleftarrows \text{Enz} + \text{AA-}\langle X \rangle + \text{AMP}$$

Fig. 1. Schematic representation of amino acid activation.

a reaction would be reversible, and if so, it might be possible to detect the overall back reaction as an incorporation of radioactive AMP into ATP that required amino acids. Using this approach, analanine-dependent incorporation of AMP into ATP was found in the "pH 5 enzyme" prepared from the low molecular weight, "soluble" fraction of rat liver homogenate. Of greatest interest was the finding that the AMP incorporation was inhibited by ribonuclease[4]. Subsequently, this alanine-dependent AMP incorporation system was reconstructed by combining a partially purified alanine-activating enzyme with low molecular weight RNA prepared from rat liver "pH 5 enzyme"[5].

In the meantime it was shown by Hoagland *et al.* [6], and by Ogata and Nohara[7] that radioactive amino acids became bound to a low molecular weight RNA in a rat-liver "pH 5 enzyme" preparation. The RNA was referred to as "soluble RNA" and is now known as "transfer RNA"[8]. Thus it became clear that the acceptor of activated amino acids was a low molecular weight RNA.

The work of Zachau, Acs and Lipmann[9], and Hecht, Stephenson and Zamecnik[10] showed that all of the activated amino acids became attached to a terminal adenosine residue in transfer RNA. Since different amino acids did not compete for the same attachment site[6,11], it seemed likely that different transfer RNA's were serving as acceptors for the different amino acids.

For a chemist, the existence of amino acid-specific, low molecular weight RNA's was very intriguing. It seemed possible that these RNA's might be small enough to permit detailed structural studies. This would be of great interest because it is the nucleotide sequences of nucleic acids that provide specificity and enable nucleic acids to carry out their many vital functions.

Isolation of yeast alanine transfer RNA

When transfer RNA's are extracted from cells, a mixture is obtained that contains at least one transfer RNA for each of the 20 different amino acids involved in protein synthesis. For detailed structural analysis, a highly purified transfer RNA was needed; therefore, in 1958, at the U. S. Plant, Soil and Nutrition Laboratory, a U. S. Department of Agriculture Laboratory at Cornell University, we set out to try to isolate an individual transfer RNA for chemical study.

Our first problem was to find a fractionation technique that was applicable to transfer RNA's Various procedures were investigated, and the Craig countercurrent distribution technique[12] was found to be promising. In collaboration with J. Apgar, B. P. Doctor and S. H. Merrill[13,14], the countercurrent distribution procedure was developed, over a period of four years, into the first generally applicable method for the fractionation of transfer RNA's. Fig. 2 shows the results of a countercurrent distribution of bulk yeast transfer RNA. Yeast transfer RNA was used because it is readily obtained in large quantity[15]. By repeated countercurrent distribution of the most active fractions obtained in Fig. 2, three of the transfer RNA's, the alanine, tyrosine, and valine RNA's, were obtained in a relatively homogeneous form and essentially free of activity as acceptors of other amino acids[14]. The results with the alanine RNA are shown in Fig. 3. The excellent correlation between the experimental curves and the calculated theoretical distribution curve, shown in Fig. 3, encouraged us to believe that the RNA was pure enough for structural analysis. Nevertheless, to undertake structural work was a gamble, since there was the possibility that the preparation might not be pure, or that it

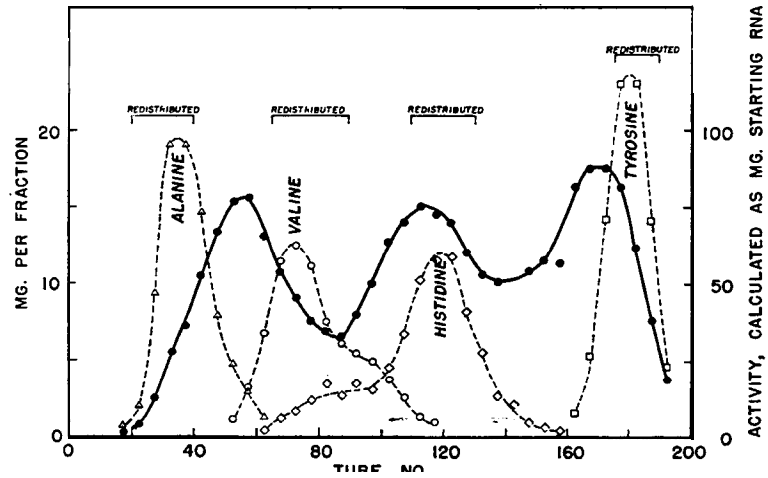

Fig. 2. 200-Transfer countercurrent distribution of 500 mg of bulk yeast transfer RNA (from ref.14).

might be a mixture of different molecular species, all of which accepted alanine. Since attempts to fractionate the material further were unsuccessful, there seemed no alternative but to gamble a few years of work on the problem hoping that the material was sufficiently pure for structural analysis. If the starting material was impure, we could expect that attempts at structural analysis would lead to hopeless confusion. Fortunately this was not the outcome.

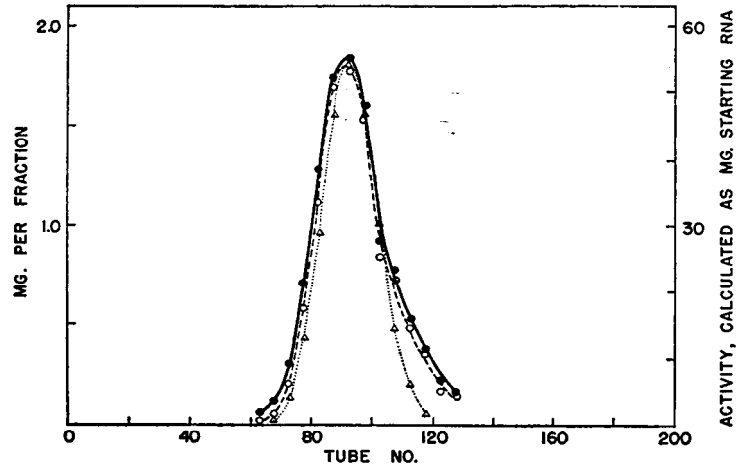

Fig. 3. 875-Transfer countercurrent distribution of redistributed alanine transfer RNA (from ref.14).

Throughout our structural studies the amount of purified alanine transfer RNA available for study was very limited. The scale of the isolation procedure was increased tenfold over that in Fig. 3, by using a large countercurrent apparatus in combination with a modified solvent system that increased the solubility of the RNA. Nevertheless, the supply of purified RNA available for individual experiments was always limited to tens of milligrams. Therefore, to the extent possible, the experiments were designed to use the minimum amount of RNA. During the three years of work on the structure of the alanine transfer RNA, we used a total of 1 g of highly purified material. This was isolated in our laboratories from approximately 200 g of bulk yeast transfer RNA, which in turn was obtained by phenol extraction of approximately 140 kg of commercial baker's yeast.

Cleavage of the RNA into small fragments

The evidence obtained in preliminary analyses indicated that the alanine transfer RNA molecule consisted of a single chain of approximately 80 nucleotide residues[16]. Therefore, in principle, structural analysis required the identification of the nucleotide residues and the determination of their sequences. Formally, the problem was analogous to determination of the sequence of approximately 80 letters in a sentence.

The experimental approach that was used involved cleavage of the polynucleotide chain into small fragments, identification of the small fragments, and then reconstruction of the original nucleotide sequence by determining the order in which the small fragments occurred in the RNA molecule. In terms of the analogy of a sentence, the approach was equivalent to breaking a sentence into words, identifying the words, and reconstructing the sequence of the letters in the sentence by determining the order of the words.

Briefly, the experiments were carried out as follows. Pancreatic ribonuclease was used to cleave the RNA chain next to pyrimidine nucleotides, to give one set of fragments in which each fragment ended in a pyrimidine nucleotide such as cytidylic acid (C-) or uridylic acid (U-). Then, takadiastase ribonuclease Tl, the enzyme discovered by Sato-Asano and Egami[17], was used, separately, to cleave the RNA chain specifically at guanylic acid (G-) residues. This gave a different set of small fragments. The individual small fragments were isolated by ion-exchange chromatography, followed by paper electrophoresis or rechromatography, where necessary. Fig. 4 shows

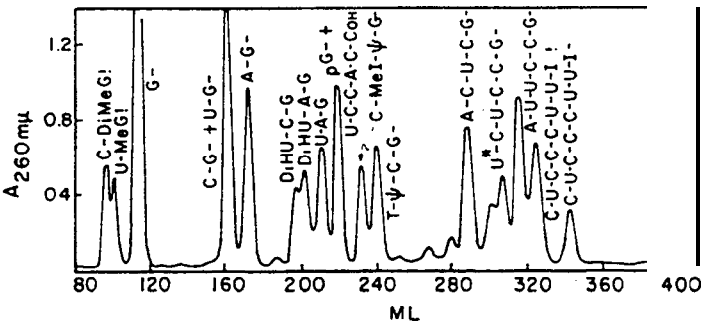

Fig. 4. Separation of ribonuclease Tl digest fragments of the alanine transfer RNA by chromatography on DEAE-cellulose. (For abbreviations see Table 1) (From refs. 23 and 31)

the pattern obtained after chromatography of the ribonuclease Tl digest on a diethylaminoethylcellulose column, using Tomlinson and Tener's procedures's with modifications by M. Marquisee and J. Apgar. Under these conditions, almost all of the different fragments obtained in the digest are separated. Each of the separated fragments was hydrolyzed with alkali, and the component mononucleotides were identified by chromatographic and electrophoretic properties and spectra. This was sufficient to determine the sequence of each of the dinucleotides, because the position of attack by each ribonuclease was known. Additional information was needed to establish the nucleotide sequences of the trinucleotides and larger oligonucleotides.

New methods of sequence determination were required in the identification of several of the larger oligonucleotides. One new method that was especially useful is outlined in Figs. 5 and 6. As indicated in Fig. 5, partial digestion of an oligonucleotide with snake venom phosphodiesterase gives a mixture of degradation products. A chromatographic pattern obtained from such a partial digest is shown in Fig. 6. Alkaline hydrolysis of the material recovered from each peak gives a nucleoside, which arises from the 3′-terminal residue

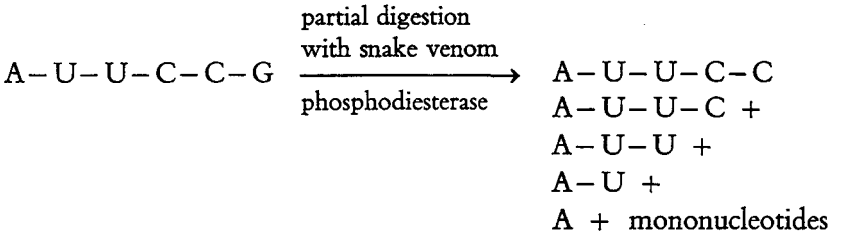

Fig. 5. Partial digestion of an oligonucleotide with snake venom phosphodiesterase.

(the right end) of the end) of the oligonucleotide in that peak. Since successive peaks in the chromatogram represent the successive stepwise degradation products, identification of the nucleosides obtained from the successive peaks gives the nucleotide sequence[19]. In the example shown in Figs. 5 and 6, the information obtained is sufficient to establish the nucleotide sequence as A - U - U- C - C - G - .

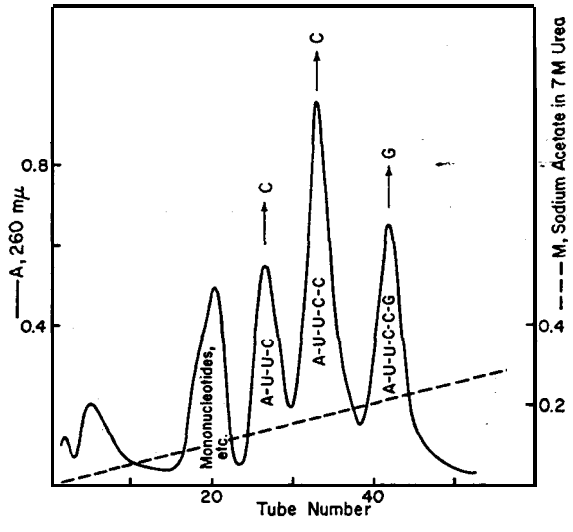

Fig. 6. Chromatographic pattern of partial digest of A - U - U - C - C - G. Recovery of the oligonucleotide from each peak followed by alkaline hydrolysis gives the nucleoside indicated (from ref. 32).

Tables 1 and 2 list the identities of the fragments obtained in the pancreatic ribonuclease and ribonuclease Tl digest, respectively[20]. Determination of the structures of all of the fragments required approximately 2.5 years. It was the work of J.T. Madison and A. Zamir, with assistance in the identification of the nucleotides from G. Everett. Especially time-consuming were the determination of the structures of the larger oligonucleotides and the identification of unusual nucleotides, particularly 1 - methylinosinic acid (Mel-) and 5,6-dihydrouridylic acid (DiHU-)[21]. (The latter nucleotide had never been found in a natural nucleic acid. It does not absorb light at 260 mμ, and, as a consequence, it is invisible in the usual procedures for detecting nucleotides.)

The presence of distinctive end groups (a free 5′-phosphate group (p) at the left end of the RNA molecule, as the structure is conventionally written, and a free 3′- hydroxyl group (OH) at the right end) established that the left end of

Table 1

Fragments obtained by complete digestion of alanine RNA with pancreatic ribonudease[2]

C_{OH}[b]	MeG–G–C–
13 C–	A–G–C–
Ψ–	A–G–DiHU–
6 U–	G–A–U–
A–C–	I–G–C–
MeI–Ψ–	G–G–T–
DiMeG–C–	G–G–DiHU–
2 G–C–	G–G–A–C–
4 G–U–	pG–G–G–C–
G–G–G–A–G–A–G–I	

[a] Abbreviations: p and - are used interchangeably to represent a phosphate residue; A, adenosine 3′-phosphate; C, cytidine 3′-phosphate; C_{OH}, cytidine (with free 3′-hydroxyl group emphasized); DiHU, 5,6-dihydrouridine 3′-phosphate; DiMeG, N^2-dimethylguanosine 3′-phosphate; 1, inosine 3′-phosphate; MeG, 1-methylguanosine 3′-phosphate; MeI, 1-methylinosine 3′-phosphate; **Ψ**, pseudouridine 3′-phosphate; T, ribothymidine 3′-phosphate; U, uridine 3′-phosphate; U*, a mixture of U, and DiHU; p!, 2′,3′-cyclic phosphate, for example: Ip!, inosine 2′,3′-cyclic phosphate.

[b] The presence of a free 3′-hydroxyl group on this fragment indicates that cytidine occupies the terminal position in the purified alanine RNA. This establishes that the terminal adenylic acid residue is missing, as it is from most transfer RNA's isolated from commercial baker's yeast. A terminal adenylic acid residue is replaced under assay conditions before the amino acid is attached.

Table 2

Fragments obtained by complete digestion of alanine RNA with takadiastase ribonuclease Tl .

9 G–	DiHU–C–G–
pG–	DiHU–A–G–
C–DiMeGp!	C–MeI–Ψ–G–
U–MeGp!	T–Ψ–C–G–
4 C–G–	A–C–U–C–G–
2 A–G–	U–C–C–A–C–C_{OH}[a]
U–G–	U*–C–U–C–C–G–
U–A–G–	A–U–U–C–C–G–
C–U–C–C–C–U–U–I–	

a See Table 1, second footnote.

the alanine transfer RNA molecule has the structure pG - G - G - C -, and the right end the structure U - C - C - A - C - C - AOH.

The presence of the unusual nucleotides, and also of certain unique sequences, gave a number of overlaps between the two sets of sequences shown in Tables 1 and 2. For example, there is only one I- in the molecule, and this is found in the sequence C - U - C - C - C - U- U - I - in the ribonuclease Tl digest and in the sequence I - G - C - in the pancreatic ribonuclease digest. These two sequences must overlap, and the overall sequence must be C - U - C - C - C - U - U - I - G - C - .

All of the information in Tables 1 and 2 is summarized in Table 3, in which the sequences are listed in such a way that all the nucleotides in the alanine RNA are accounted for in 16 sequences that total 77 nucleotide residues.

Table 3

Sequences that account for the nucleotide residues in the alanine RNA[a]

–G–G–C–, G–U–G–, U–MeG–G–C–, G–C–,
-U–A–G–, DiHU–C–G–, G–DiHU–A–G–,
-G–, C–DiMeG–, C–U–C–C–C–U–U–I–G–C–,
. . . . .
I–Ψ–, G–G–G–A–G–A–G–U*–C–U–C–C–G–,
-T–Ψ–C–G–, A–U–U–C–C–G–, G–A–C–U–C–G–,
-C–C–A–C–C–A_{OH}

[a] Dotted line separates sequences present in one half of the molecule from those present in the other half.

Cleavage of the RNA into large fragments

Once the 16 sequences shown in Table 3 were known, with the positions of the two end sequences established, the structural problem became one of determining the positions of the 14 intermediate sequences. This was done by isolating a number of large fragments from the RNA. In a crucial experiment it was found by J. R. Penswick that very brief treatment of the RNA with ribonuclease Tl at 0°C in the presence of magnesium ion splits the molecule at one position[22]. The two halves of the molecule could be separated by chromatography (Fig. 7). Subsequent digestion of the separated half molecules with ribonuclease Tl, followed by chromatographic analysis (Fig. 8), established that the sequences listed above the dotted line in Table 3 were present in the left half of the molecule and the remaining sequences were present in the right half[22].

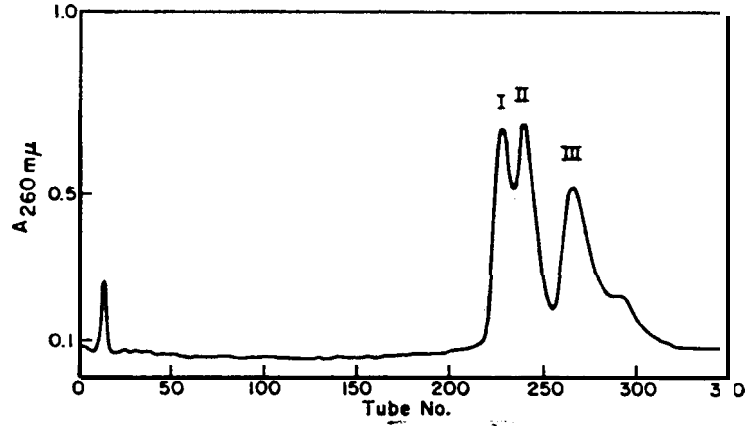

Fig. 7. Chromatographic separation of two large fragments (I and II) obtained by very limited digestion of the alanine transfer RNA with ribonuclease Tl at 0° in the presence of magnesium ion (from ref. 22).

Using somewhat more vigorous, but still limited treatment of the RNA with ribonuclease Tl, we then obtained, with J. Apgar, a number of additional large fragments. To determine the structures of the large fragments, each large fragment was degraded completely with ribonuclease Tl, the digest was chromatographed to give two or more of the ribonuclease Tl peaks already identified in Fig.4, and these known sequences were put together, one after

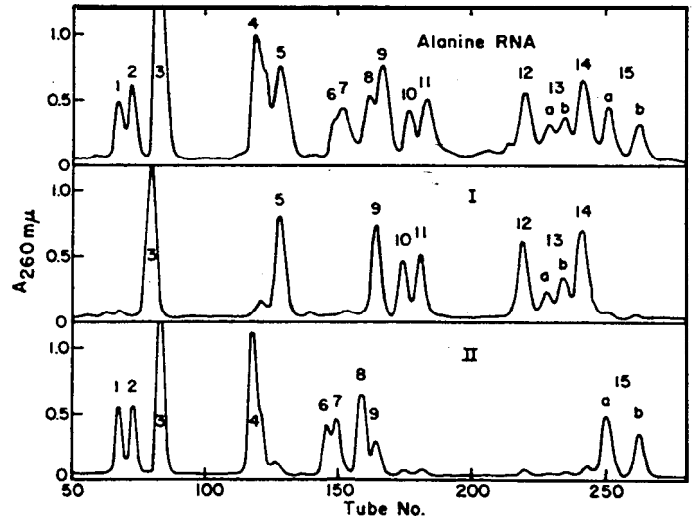

Fig. 8. Chromatography of ribonuclease Tl digests of the alanine transfer RNA and large fragments I and II from Fig. 7 (from ref. 22).

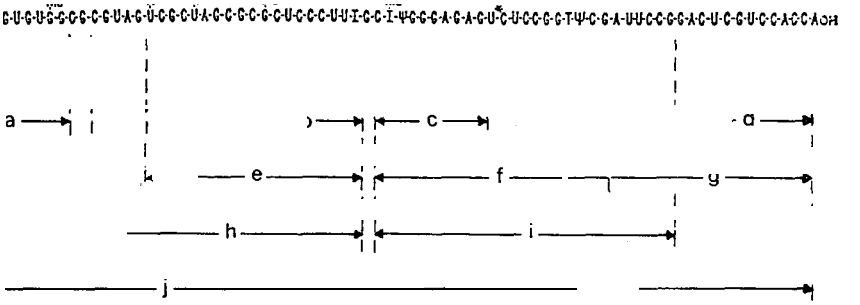

Fig. 9. The nucleotide sequence of the yeast alanine transfer RNA is shown at the top. Large oligonucleotide fragments isolated and used to reconstruct the complete sequence are indicated below (from ref.25).

another, until the complete nucleotide sequence of the large fragment was known. The sequences that were determined are indicated in Fig. 9 by the letters a to k[23,24].

The approach used in reconstructing the long sequences can be illustrated by considering two fragments in detail.

The chromatographic analysis of a complete ribonuclease Tl digest of fragment *d* is shown in Fig. 10. The presence of U - C - C - A - C - COH indicates that fragment d is from the right end, the 3′-end, of the molecule. Therefore, the A - C - U - C - G - sequence must be to the left of this, and the sequence of d is known[23].

The chromatographic analysis of a ribonuclease Tl digest of fragment a is shown in Fig. 11. The analysis indicates that fragment a is composed of U - MeG -, 3G -, C - G -, U - G -, and pG -. The presence of pG - establishes that fragment a is from the left end of the RNA molecule. Since it is

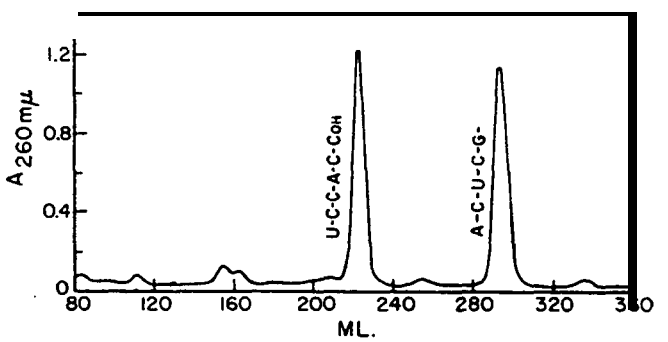

Fig.10. Chromatography of complete ribonuclease Tl digest of fragment d (from ref.23).

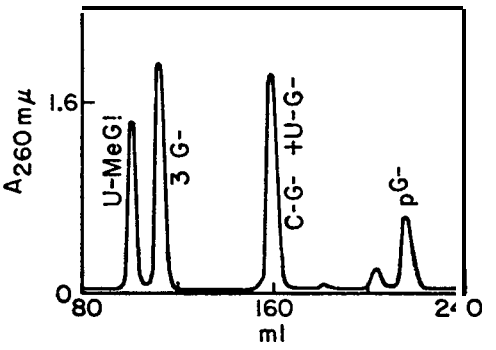

Fig.11. Chromatography of complete ribonuclease Tl digest of fragment a (from refs. 23 and 31).

already known (Tables 1 and 3) that the terminal sequence at the left end of the RNA is pG - G - G - C -, the positions of two of the three G -'s and the C - G - are known, and the terminal five nucleotides must be pG - G - G - C - G -. The positions of the U - G -,U - MeG -, and G - are established by the following information. It is known (Table 3) that the U - MeG - is present in the RNA in the sequence U - MeG - G - C. Since there is only one C in fragment a, and its position has already been established, fragment a must terminate before the C of the U - MeG - G - C - sequence. Therefore, the U- G- must be to the left of the U- MeG-, and the structure of fragment a can be represented as pG - G - G - C - G - ···U - G - ···- U - MeG -, with one G - remaining to be placed. If the G - is placed to the left or the right of the U - G - in this structure, there will be G - G - U - sequence in the RNA. Since any G - G - U- sequence would appear in a pancreatic ribonuclease digest, and such a sequenceis not found (Table 1), the remaining G - must be to the right of the MeG - , and the sequence of fragment a is pG - G - G - C - G - U - G - U - MeG - G[23].

The structural proofs for the other large fragments were carried out in a similar fashion[24]. Some of these proofs were straightforward; others were difficult. Eventually, the analyses of the large fragments furnished sufficient information to establish the sequences of the halves of the RNA molecule (fragments j and k in Fig. 9). Since the terminal sequences of the RNA were already known, the halves could be joined in only one way, to give the I - G - C - sequence which was known to be present in the RNA (Table 1), and the complete nucleotide sequence of the yeast alanine transfer RNA is that shown in Fig. 9[25].

This is the first nucleotide sequence known for a nucleic acid. Also, it can be said that the sequence gives, with appropriate modifications for DNA, the first nucleotide sequence of a gene. This would be the sequence of the gene that determines the structure of the alanine transfer RNA in yeast cells.

It was, of course, tremendously satisfying to be able to solve each experimental problem as it arose, and eventually be able to complete the nucleotide sequence. The satisfaction was increased by the fact that we were able to work with the alanine transfer RNA from discovery to isolation to structural analysis. In these times of highly competitive research, few scientists have the satisfaction of carrying through a research problem that takes 9 years. Without minimizing the pleasure of receiving awards and prizes, I think it is true that the greatest satisfaction for a scientist comes from carrying a major piece of research to a successful conclusion.

Three-dimensional structure

When a problem is solved, one's attention turns to other problems. With the complete nucleotide sequence of the alanine transfer RNA established, we became concerned with other questions about the alanine RNA structure. One question of particular interest has to do with the interaction of the transfer RNA with a messenger RNA. Speculation suggests that the three-dimensional structure of a transfer RNA, in the presence of the magnesium ion under conditions suitable for protein synthesis, should have the coding triplet of nucleotides, the anticodon, exposed in a way that will permit it to interact with a triplet of nucleotides, the codon, in the messenger RNA[26]. The sequence that constitutes the anticodon in the alanine transfer RNA is the sequence I - G - C, present in the middle of the molecule and including the linkage that is so sensitive to attack by ribonuclease Tl. One arrangement of the RNA chain, suggested by E. B. Keller and by Penswick, has the I - G - C - sequence in an exposed position and also has very interesting symmetry. This "cloverleaf" arrangement is shown in Fig. 12[25]. In drawing this arrangement, it was assumed that there would be Watson-Crick-type pairing of A to U and G to C in the double-stranded regions and the unpaired regions would form loops as suggested by Fresco, Alberts and Doty[27]. The strongest evidence for the "cloverleaf" arrangement of the secondary structure of transfer RNA's comes from the finding that all of the transfer RNA sequences that have been determined since 1965 fit the same type of base-pairing arrange-

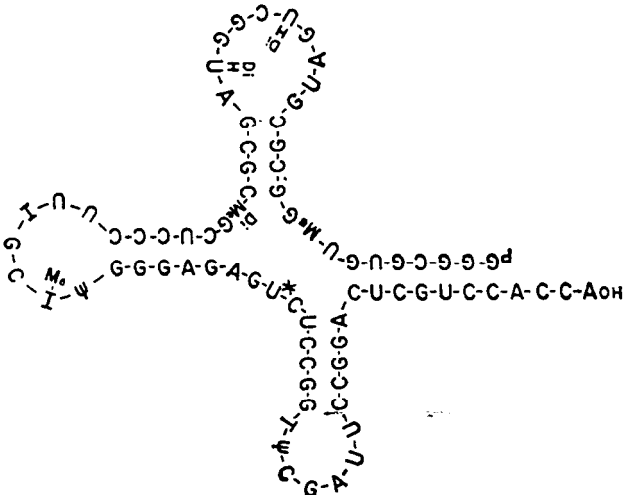

Fig.12. Suggested secondary structure of the alanine transfer RNA (from ref.25).

ment. There are now 12 of these sequences and they have come from structural studies in many different laboratories[28]. In all instances the anticodon sequence is found at the same position in the middle loop. The "cloverleaf" arrangement can be only a partial description of the three- dimensional structural. This is clear from chemical and enzymatic studies, which indicate that the molecule is folded in some way[29]. However, details of the folding are not clear. Some further information can no doubt be obtained by chemical and enzymatic probing, but it seems likely that proof of the three-dimensional structure of a transfer RNA will wait for X-ray analysis[30].

That then is our story of the alanine transfer RNA. It all followed quite naturally from taking a sabattical leave. I strongly recommend sabbatical leaves.

Acknowledgment

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