STRUCTURAL ANALYSIS OF NONRADIOACTIVE RNA BY POSTLABELING: THE PRIMARY STRUCTURE OF BAKER'S YEAST $tRNA^{Leu}_{CUA}$

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SUMMARY. The sequence of $tRNA^{Leu}_{CUA}$ from baker's yeast was determined by application of novel postlabeling methods. Oligonucleotides in complete and partial RNase digests (A, T_1, and U_2) were resolved by chromatography on polyethyleneimine-cellulose thin layers. Nucleotide sequences were elucidated by $NaIO_4$/phosphatase or snake venom phosphodiesterase/phosphatase digestion and tritium postlabeling. A ribose-methylated constituent (Gm) was identified by 5'-terminal [^{32}P]-labeling. $tRNA^{Leu}_{CUA}$ is the first biological macromolecule to be sequenced according to a postlabeling scheme.

Two novel, highly sensitive tritium postlabeling methods for sequence analysis of nonradioactive ribooligonucleotides were described recently (1,2). These methods are several orders of magnitude more sensitive than the classical spectrophotometric methods and do not require in vivo labeling with ^{32}P. Here we report briefly results obtained by applying these methods to the structural analysis of a $tRNA^{Leu}$ species from baker's yeast. Details of this work will be published elsewhere.

MATERIALS AND METHODS. Crude tRNA was prepared from baker's yeast by phenol extraction (3,4). $tRNA^{Leu}_{CUA}$ was purified by chromatography on 2 successive columns of benzoylated DEAE-cellulose (5), first in the presence of 10 mM $MgCl_2$ and then in the absence of $MgCl_2$ (6). A small amount of $tRNA^{Leu}_{UUG}$ (7) present in the $tRNA^{Leu}_{CUA}$ fraction from the second column was removed by passing the sample through a column of Sephadex G-100 after subjecting the tRNA to conditions under which $tRNA^{Leu}_{UUG}$ undergoes reversible denaturation (8). The tRNA preparation used in the sequencing work accepted 1590 pmoles of leucine per $O.D._{260}$ unit.

Conditions for complete digestion by RNases were as detailed by Brownlee (9). Partial digestion with RNase T_1 was a scaled-down version of a published procedure (10) except that 50 units of enzyme were used per $O.D._{260}$ unit of RNA and incubation was for 100 min at 0°. After phenol extraction, the digest was resolved by chromatography on a DEAE-cellulose

column at acidic pH in the presence of 7 M urea (11). Two peaks were obtained consisting of the respective half molecules (see below). Digestion with RNase U_2 (12) was carried out under conditions similar to those described by Takemura et al. (13).

Digests (3 $O.D._{260}$ units) were resolved by 2-dimensional anion-exchange thin-layer chromatography on PEI-cellulose, using Tris-HCl or LiCl gradients in 8.5 M urea (pH 8.0) (1) for the first, and lithium formate gradients (14) in 7.5 M urea (pH 3.5) for the second dimension. Compounds were located under UV light, extracted with electrolyte solutions and purified with phosphocellulose (15). The extracts were concentrated and subjected to tritium sequence analysis (1,2).

Oligonucleotides containing ribose-methylated nucleosides (Nm) were characterized by the following steps: 1) digestion with RNase T_2 to Nm-N- and N-; 2) treatment with alkaline phosphatase; 3) inactivation of phosphatase (16); 4) phosphorylation of 5'-hydroxyl of Nm-N by treatment with [γ-^{32}P]ATP and polynucleotide kinase (17); 5) digestion of [^{32}P]pNm-N formed in step 4 with snake venom phosphodiesterase to [^{32}P]pNm and pN; 6) chromatographic identification of [^{32}P]pNm by thin-layer cochromatography in the presence of suitable markers. [^{32}P]pGm marker was prepared by subjecting Gm-A-A-Y-A-Ψ-, which was obtained by RNase A digestion of baker's yeast $tRNA^{Phe}$ (18), to this procedure.

RESULTS. Fig. 1 shows the sequence of $tRNA^{Leu}_{CUA}$ written in linear form and also the fragments obtained by complete digestion of the RNA with RNase T_1 (digest T) and RNase A (digest A). The arrow indicates the site of cleavage obtained by partial digestion with RNase T_1. Fragments formed upon digestion of the half molecules with RNase A (digests A(1) and A(2)) and RNase U_2 (digests U(1) and U(2)) are shown in the lower part of Fig. 1. Production of the halves facilitated ordering the oligonucleotides. Fragment A8 (A-G-m^1G-C-), which was present at a molar ratio of 1 in digest A, was not detected in the corresponding digests of the halves (A(1) and A(2)). Instead, digest A(1) contained A-G-, and m^1G-C- was found in digest A(2) (Fig. 1). Since these dinucleotides were not present in digest A it may be concluded that cleavage occurred between G and m^1G in the tetranucleotide sequence -A-G-m^1G-C-.

The sequence from position 18 to the 3'-terminus of the 5'-half (position 37) may be deduced on the basis of digests T, A, and A(1) alone. Positions 1 - 14 can be deduced from fragments A1, T3, and U(1)2 since there is only 1 -G-U- sequence in the molecule (digests A and A(1)). Fragment A3 extends this sequence to position 16. Position 17 must be occupied by Gm because tritium base composition analysis (19) showed the RNA to contain 4 D residues, all located in the 5'-half, so that fragments

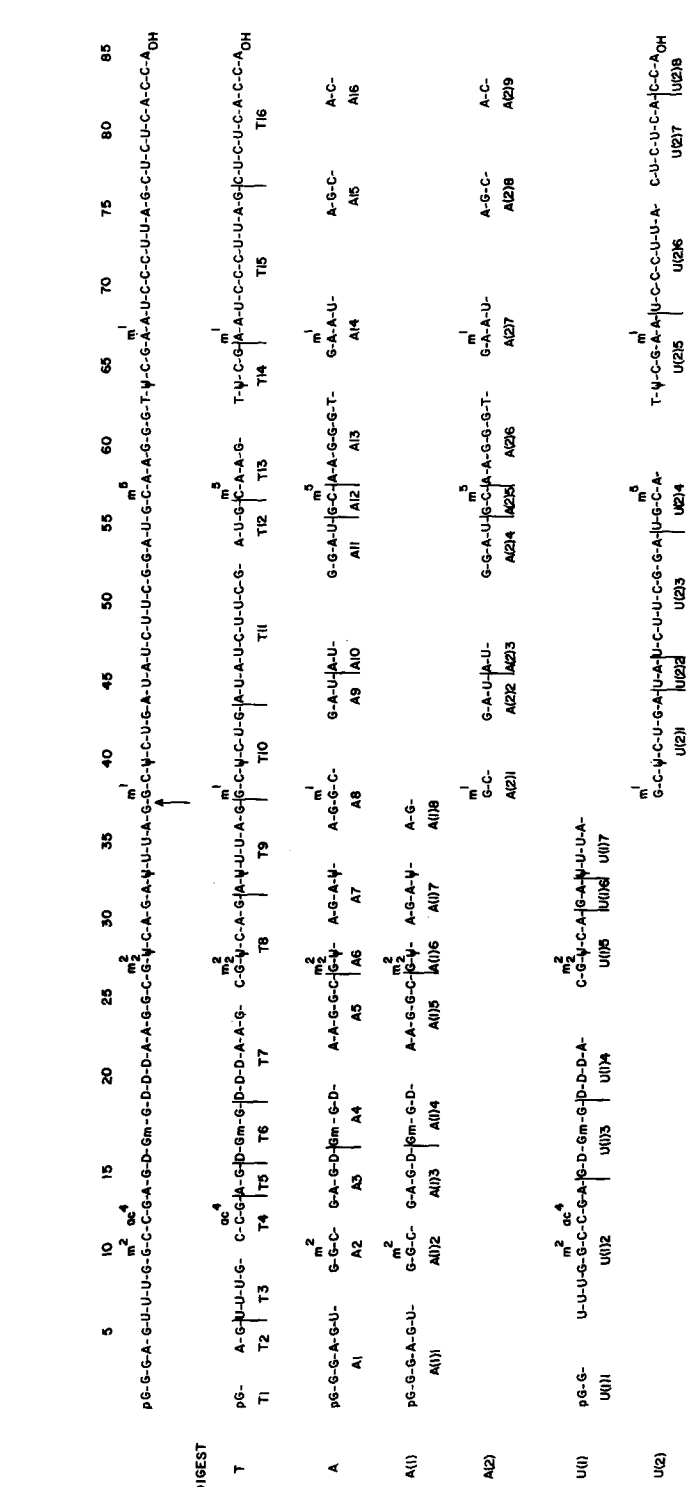

Fig. 1 Nucleotide sequence of baker's yeast $tRNA^{Leu}_{CUA}$ in linear form (top). Below, fragments obtained by digestion with various enzymes (see text). The arrow indicates the site of cleavage upon partial digestion of the tRNA with RNase T1.

T6 and A4, which were found to be present each at a molar ratio of 1, can only occupy positions 16 - 18 and 17 - 19, respectively. The identity of Gm was established by the procedure outlined under Materials and Methods.

For ordering the fragments in the 3'-half, oligonucleotides U(2)1 and U(2)3 were essential. Since analysis of digest A(2) showed the presence of 2 -G-A-U- sequences (A(2)2 and A(2)4) it could be concluded that both U(2)1 and U(2)3 have U-adjacent to their respective 3'-terminal -G-A- sequences. The connection between U(2)1 and U(2)3 is provided by T11, thus establishing the sequence from position 38 to 55. Position 56 must be occupied by G on the basis of T12 since there are only 2 -G-A-U- sequences in the molecule one of which occupies positions 43 - 45. Fragment U(2)4 provides evidence that T13 directly follows T12 in the sequence. T13, A13, T14, and U(2)5 provide necessary overlaps so as to extend the sequence of the 3'-half to position 68. The only fragments not completely accounted for so far are A14, A15, A16, T15, and T16. A14 and U(2)5 show that T15 directly follows T14, and A15 shows that T16 follows T15, thus establishing the total sequence of the 3'-half.

DISCUSSION. Fig. 2 shows the sequence of the RNA arranged in the familiar cloverleaf form (20). The molecule exhibits no unusual features, e.g., modified nucleosides occupy positions expected from other tRNA sequences. The sequence is remarkably similar to that of $tRNA^{Leu}_{UUG}$ (7): of a total of 85 nucleotides in both RNAs, 66 are identical as indicated in Fig. 2. A notable difference exists in the respective anticodon sequences, i.e., -U-A-G- in the present RNA and -m^5C-A-A- in $tRNA^{Leu}_{UUG}$. This is only the second instance to date in which it is possible to compare, in the same organism, the sequences of 2 isoaccepting tRNAs which differ in the 3'-terminal base of the anticodon, i.e., the base interacting with the first letter of the codon triplet. In the other example, $tRNA^{Arg}$ from brewer's yeast (21,22), 49 out of 76 nucleotides are the same in the 2 RNAs. The identical nucleotides, in the case of $tRNA^{Arg}$, are also clustered around the "center" of the cloverleaf model, including the variable loop. Such long common sequences appear to indicate that large continuous portions of the tRNA structure may be

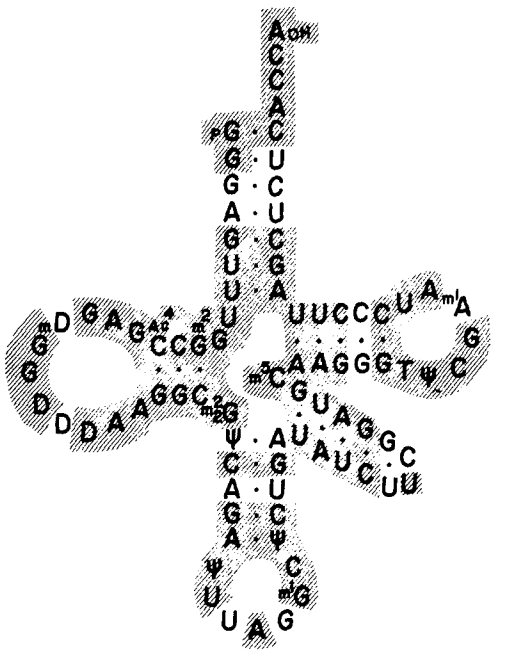

Fig. 2 Nucleotide sequence of baker's yeast $tRNA^{Leu}_{CUA}$ in the cloverleaf form. Hatched areas indicate identical nucleotides in $tRNA^{Leu}_{CUA}$ and $tRNA^{Leu}_{UUG}$.

recognized as specific for a particular amino acid by the diverse cellular elements that interact with tRNA. The enzyme recognition site may consist of a more restricted number of nucleotides, as shown for yeast phenylalanyl-tRNA synthetase (23). Very little homology exists between the 2 yeast $tRNA^{Leu}$ species and $tRNA^{Leu}_1$ and $tRNA^{Leu}_2$ from E. coli (24).

In contrast to $tRNA^{Leu}_{UUG}$ ($tRNA^{Leu}_3$), the present tRNA cannot be denatured by heating in the absence of Mg^{++}. This is consistent with a model of the denatured conformer of $tRNA^{Leu}_{UUG}$, which postulates the formation of a new helix by pairing 5 bases from the anticodon loop/stem area (positions 31 - 35) with 5 bases from the TψC loop/stem area (positions 66 - 70) (25). Because of the presence, in $tRNA^{Leu}_{CUA}$, of U instead of m^5C in position 35 and of m^1A instead of A in position 67, $tRNA^{Leu}_{CUA}$ cannot form such a stable helix. It may correspond to either one of 2 non-denaturable yeast $tRNA^{Leu}$ species ($tRNA^{Leu}_1$ and $tRNA^{Leu}_2$) separated by countercurrent distribution (8).

We conclude on a technical note. The tritium derivative procedures (1,2), as used in the present investigation, make it

possible to characterize the fragments in a complete RNase digest derived from about 3 $O.D._{260}$ units. We have used a total of about 6 $O.D._{260}$ units of tRNA to deduce the sequences of the fragments in digests T and A (Fig. 1). On the basis of this information, and taking into account the known structural features of tRNA, we were able to write down a tentative sequence, which was subsequently confirmed by the analysis of the half molecules. With further refinement of the methods, it should be possible to elucidate the primary structure of tRNAs with as little as 1 - 5 $O.D._{260}$ units of nonradioactive RNA. Postlabeling methods may thus open up the possibility to characterize transfer and other RNAs that cannot readily be labeled in vivo to the high specific radioactivity required for sequence analysis (26) and are difficult to obtain in amounts sufficient for sequence analysis by spectrophotometric methods, for example, RNAs in human and other mammalian tumor tissues.

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REFERENCES

1. Randerath, K., Randerath, E., Chia, L.S.Y., Gupta, R.C., and Sivarajan, M. (1974) Nucl. Acids. Res. 1, 1121-1141.
2. Sivarajan, M., Gupta, R.C., Chia, L.S.Y., Randerath, E., and Randerath, K. (1974) Nucl. Acids Res. 1, 1329-1341.
3. Monier, R., Stephenson, M.L., and Zamecnik, P.C. (1960) Biochim. Biophys. Acta 43, 1-8.
4. Holley, R.W. (1963) Biochem. Biophys. Res. Comm. 10, 186-188.
5. Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G.M. (1967) Biochemistry 6, 3043-3056.
6. Chang, S.H., Kuo, S., Miller, N.R., and Hawkins, E.R. (1973) Fed. Proc. 32, 618 (Abstr. 2272).
7. Chang, S.H., Kuo, S., Hawkins, E.R., and Miller, N.R. (1973) Biochem. Biophys. Res. Comm. 51, 951-955.
8. Lindahl, T., Adams, A., and Fresco, J.R. (1966) Proc. Natl. Acad. Sci. U.S. 55, 941-948.
9. Brownlee, G.G. (1972) Determination of Sequences in RNA, North-Holland/American Elsevier, Amsterdam and New York.
10. RajBhandary, U.L., and Chang, S.H. (1968) J. Biol. Chem. 243, 598-608.
11. Rushizky, G.W., and Sober, H.A. (1964) Biochem. Biophys. Res. Comm. 14, 276-279.
12. Arima, T., Uchida, T., and Egami, F. (1968) Biochem. J. 106, 609-613.
13. Takemura, S., Kasai, H., and Goto, M. (1974) J. Biochem. 75, 1169-1172.

14. Mirzabekov, A.D., and Griffin, B.E. (1972) J. Mol. Biol. 72, 633-643.
15. Randerath, E., and Randerath, K. (1967) J. Chromatog. 31, 485-499.
16. Min-Jou, W., and Fiers, W. (1969) J. Mol. Biol. 40, 187-201.
17. Richardson, C.C. (1971) in: Cantoni, G.L., and Davies, D.R. (eds.) Procedures in Nucleic Acid Research, vol. 2, pp. 815-828, Harper and Row, New York.
18. RajBhandary, U.L., Faulkner, R.D., and Stuart, A (1968) J. Biol. Chem. 243, 575-583.
19. Randerath, E., Yu, C.-T., and Randerath, K. (1972) Anal. Biochem. 48, 172-198.
20. Holley, R.W., Apgar, J., Everett, G.A., Madison, J.T., Marquisee, M., Merrill, S.H., Penswick, J.R., and Zamir, A. (1965) Science 147, 1462-1465.
21. Kuntzel, B., Weissenbach, J., and Dirheimer, G. (1972) FEBS Lett. 25, 189-191.
22. Weissenbach, J., Martin, R., and Dirheimer, G. (1972) FEBS Lett. 28, 353-355.
23. Roe, B., and Dudock, B. (1972) Biochem. Biophys. Res. Comm. 49, 399-406.
24. Blank, H.-U., and Söll, D. (1971) Biochem. Biophys. Res. Comm. 43, 1192-1197.
25. Kearns, D.R., Wong, Y.P., Chang, S.H., and Hawkins, E.R. (1974) Biochemistry 13, 4736-4746.
26. Sanger, F., Brownlee, G.G., and Barrell, B.G. (1965) J. Mol. Biol. 13, 373-398.