COMMUNICATIONS

³²P-Labeling of Haemoglobin Messenger and Other Reticulocyte Ribonucleic Acids with Polynucleotide Phosphokinase *in vitro*

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The fractionation procedures developed by Sanger, Brownlee & Barrell (1965) for the separation of nucleotides derived from uniformly ³²P-labelled nucleic acids have already permitted the determination of the sequence of some low-molecular-weight RNA species. These methods may be extended to the study of nucleic acids that cannot be sufficiently labelled *in vivo* or in tissue culture by the use of polynucleotide phosphokinase (Richardson, 1965; Murray & Wu, 1968; Szekely & Sanger, 1969), which catalyses the transfer of orthophosphate from [γ -³²P]ATP to the 5'-hydroxyl termini of oligo- and poly-nucleotides (Richardson, 1965; Novogrodsky & Hurwitz, 1966). We have studied the optimum conditions of this reaction for ³²P-labelling of ribonuclease digestion products and applied the technique to the study of reticulocyte RNA isolated from polyacrylamide gels (Labrie, 1969).

RNA stained with Methylene Blue was extracted by homogenization of the individual or pooled gel fraction. RNA (0.1–0.5 μ g.) was incubated successively with T-1 or pancreatic ribonuclease and bacterial alkaline phosphatase. A large proportion of the enzymic activity was removed by extracting with aqueous phenol. The nucleotides were then ³²P-labelled at their 5'-hydroxyl termini by incubating with polynucleotide kinase and a two- to three-fold excess of [γ -³²P]ATP. Unchanged ATP was removed with myosin. The nucleotides were then fractionated by the standard two-dimensional procedures (Sanger *et al.* 1965).

We have shown previously that 10s reticulocyte RNA labelled *in vivo* had a specific 'fingerprint' different from those of 18s and 29s RNA. By labelling with polynucleotide kinase, we have obtained 'fingerprints' of the minor bands previously observed. The pattern of 15s and 11s RNA indicates that they are probably degradation products of 18s RNA, and 9s RNA probably originates from 29s RNA. A specific pattern was again observed for 10s RNA similar to that found by labelling *in vivo*.

The double bands observed for 5s and 7s RNA on 7.5% polyacrylamide gels gave identical 'fingerprints', indicating that they represent different conformations of the same molecule. The complete T-1 ribonuclease digests of 5s RNA from

rabbit reticulocyte and from rat pituitary were analysed by partial hydrolysis with venom phosphodiesterase and found to be identical with those published for human KB-cell carcinoma (Forget & Weissman, 1967).

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5s Ribosomal Ribonucleic Acid from Two Mouse Cell Lines Gives 'Fingerprints' Identical with Human 5s Ribosomal Ribonucleic Acid

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5s Ribosomal RNA labelled with ³²P was isolated from Landschütz cells (a mouse ascites-tumour line) and LS cells (a line derived from mouse subcutaneous tissue) in culture. The RNA was 'fingerprinted' by using the techniques developed by Sanger, Brownlee & Barrell (1965). The 'fingerprints' of T-1 ribonuclease, T-1 ribonuclease plus phosphatase, and pancreatic-ribonuclease digests show no detectable differences in sequence or amount of oligonucleotides from those determined for human KB-cell 5s ribosomal RNA by Forget & Weissman (1967), with the exception of a possible difference in the proportions of the two 3'-chain termini -C-U-U-OH and -C-U-U-U-OH.

For a molecule of this size identical 'fingerprints' are very strong evidence for identical sequence. It is possible that this invariance points to a very exact function for 5s RNA, demanding for molecular reasons this particular nucleotide sequence and none other. It would appear that the 5s ribosomal RNA system, with an implied gene multiplicity for an invariant and non-diverging small RNA of known base sequence, would prove an interesting system for hybridization experiments with mammalian material.

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A Defect in the 40s Ribosomal Subunit after Hypophysectomy of the Rat

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Hypophysectomy of the rat diminishes, and growth-hormone injection stimulates, the ability of isolated liver ribosomes to incorporate amino acids into protein in the absence or presence of excess of polyuridylic acid (Korner, 1968). We report the results of experiments designed to detect which of the ribosomal subunits has been effected by hypophysectomy.

Rat liver ribosomes were prepared by a method involving precipitation with Mg^{2+} ions (Takanami, 1960) to remove ferritin, which obscures the large ribosomal subunit on sucrose density gradients. The ribosomes were dissociated to subunits by adjusting the K^+ ion concentration of the medium to 0.8M and warming to 30° for 5 min. (Martin & Wool, 1968). The dissociated ribosomes were layered on 7–30% linear sucrose gradients containing 0.8M-KCl. After centrifugation at 2° in an MSE 3 × 20ml. rotor for 5 hr. at 100000g, the gradients were analysed at 260nm. The fractions corresponding to subunits were collected. Hybrid ribosomes were formed from some of them by mixing the 60s and the 40s subunits in the E_{260} ratio 2.5:1. The mixture was dialysed overnight to remove excess of KCl and sucrose, and the hybrids and the subunits were assayed for their ability to catalyse polyphenylalanine synthesis in the presence of polyuridylic acid. The conditions of incubation were as described by Munro, Jackson & Korner (1964).

The reconstituted ribosome made from the 60s and 40s subunits of normal liver ribosomes, and the hybrid made from a 40s normal subunit with a 60s subunit from hypophysectomized rat liver showed about equal activity. Any hybrid containing the 40s subunit from hypophysectomized rat, however, had less ability to incorporate [^{14}C]-phenylalanine into acid-precipitable material than hybrids containing the normal 40s subunit.

Martin & Wool (1968) found that ribosomes from muscle of diabetic rats, which are less active than normal ones, have a defect in the 60s subunit. Our results show that the defect resulting from hypophysectomy is in the 40s subunit.

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Conversion of 4-Thiouracil in Amino Acid-Transfer Ribonucleic Acid into N^4 -Methylcytidine for Sequence Analysis

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Although sequence analysis of oligonucleotides containing 4-thiouracil is hampered by the chemical reactivity of the sulphur atom, this feature can be used to convert 4-thiouracil into a stable pyrimidine which can, if desired, be selectively labelled with ^{14}C . OsO_4 is known to oxidize 4-thiouridine beyond the disulphide and the oxidation product(s) react readily with ammonia to form cytidine (Burton, 1967). With methylamine, the principal final product is N^4 -methylcytidine and the reaction can be applied to introduce [^{14}C]methylamino groups into amino acid-transfer RNA (t-RNA*). Analogous reactions of periodate-oxidized 4-thiouridine have been discovered independently by Ziff & Fresco (1968).

Typical conditions are to dissolve 1mg. of t-RNA in 70 μ l. of a solution containing 20mM- $[^{14}C]$ -methylamine (specific radioactivity 10c/mole) and 16mM-HCl. OsO_4 (10 μ l. of an aq. 90mM solution) is then added and the mixture is incubated for 1 hr. at 37°. Residual osmium compounds are reduced by incubation with 10 μ l. of mercaptoethanol for 10 min. The RNA is precipitated by ethanol and freeze-dried. Unfractionated t-RNA from *Escherichia coli* gives several radioactive oligonucleotides after digestion with ribonucleases and separation by the two-dimensional systems of Sanger, Brownlee & Barrell (1965). On complete degradation to nucleotides, N^4 -methylcytosine nucleotide was the only radioactive compound detected. The pancreatic nuclease digests support the conclusion of Lipsett (1968) in indicating that most of the 4-thiouridine is linked through its 5'-hydroxyl group to a purine nucleotide; nevertheless, Py-A-G-thioU and Py-G-A-thioU are rare sequences. In T-1 ribonuclease digests of the modified RNA, labelled nucleotides resembling (A, N^4 -methylC)-G are the most common. Purified preparations of phenylalanine t-RNA gave single radioactive spots in both T-1 and pancreatic-ribonuclease digests, and their behaviour on partial

* Abbreviations: t-RNA, transfer RNA; Py, pyrimidine; thioU, 4-thiouridine; N^4 -methylC, N^4 -methylcytidine.