Studies on the Secondary Structure of Nuclear Ribonucleic Acids

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Rat liver nuclei were separated into two fractions, the nucleolus and the nucleoplasm, by a combined salt-enzymic extraction. RNA was purified from both sources and analysed on sucrose density gradients. In both fractions a prominent heterogeneous RNA class with mean sedimentation coefficient of 18S was found. This material was analysed by measuring the rate of reaction with formaldehyde, the ultraviolet absorbance-temperature profile, the spectrophotometric observation of conformational changes as a function of pH, the spectrophotometric titration of uracil and guanine residues and the effect of both temperature and ionic strength on the spectrophotometric titration of cytosine residues. Nucleoplasmic 18S RNA fraction exhibited, on heating and also by adjustment of the pH to 2.5, a hyperchromicity of about 16%, close to that observed, in control experiments, for ribosomal RNA (22%). Titration of cytosine residues in solutions containing 1mM-NaCl and 0.1M-NaCl yielded pK values equal to 4.41 and 3.84 respectively. These results suggest that this RNA fraction is composed of structurally complex polymers. The hypochromicity of the nucleolar 18S RNA fraction determined by heating or adjusting the pH to 2.5, was not greater than 6% of the initial value. The rate of reaction with formaldehyde was 88% of that observed for the hydrolysed 18S fraction which suggested only 12% hydrogen bonding. pK values for uracil and guanine residues were 10.1 and 10.05 respectively. Titration of cytosine residues yielded a pK of 4.10, which was found to be independent of temperature and ionic strength variations.

Most of the assumptions put forward for mRNA by Jacob & Monod (1961) were verified by techniques that did not require its separation from the bulk of cellular RNA.

For this reason, little knowledge has been gained on mRNA structure, since the physico-chemical methods, widely applied to the elucidation of the conformation of other polynucleotides such as DNA, tRNA or rRNA, require that the compound to be examined can be isolated in a reasonably pure form.

The discovery that synthetic polyribonucleotides could direct amino acid incorporation *in vitro* (Nirenberg & Matthaei, 1961) when added to messenger-depleted ribosomes, permitted the use of these molecules as models for the determination of the structure of informational polyribonucleotides.

Thus, it was shown that poly(U), which is completely devoid of secondary structure above 10°C (Michelson *et al.*, 1967), or poly(A) and poly(C), which are stacked but not hydrogen-bonded polymers (Fasman *et al.*, 1964; Brahms *et al.*, 1966; Felsenfeld & Miles, 1967), were active in stimulation of amino acid-incorporating activity, whereas hydrogen-bonded polymers such as DNA, tRNA and rRNA were ineffective, unless previously denatured and incubated in the presence of neomycin (McCarthy & Holland, 1966; Holland *et al.*, 1966).

Further, the ability of poly(U) to direct the synthesis of polyphenylalanine is progressively lost as increasing amounts of poly(G) are added to the incubation medium, which shows that an inverse relationship exists between the informational power and hydrogen bonding (Singer *et al.*, 1963).

On the other hand, the structure of viral ribonucleic acids presents a completely different picture. Studies on tobacco mosaic virus (Doty *et al.*, 1959; Boedtker, 1960), turnip yellow mosaic virus (Ofengand & Haselkorn, 1962; Cox, 1966a), reovirus, and sweet clover wound tumour virus (Gomatos & Tamm, 1963), bacteriophage T₄ (Bautz, 1963), and bacteriophage R₁₇ (Gesteland & Boedtker, 1964), showed that hydrogen bonding is always present and is necessary to ensure correct initiation of translation (Steitz, 1969; Adams *et al.*, 1969; Lodish & Robertson, 1969).

Physical studies were made possible on another model, by the demonstration, in isolated rat liver nucleoli, of a heterogeneous RNA, with a mean sedimentation coefficient of 18S, which was DNA-like in its base composition and was active in the stimulation of amino acid incorporation *in vitro* (M. Brentani *et al.*, 1967; Brentani & Brentani, 1969), and by the development of a technique that permits the fractionation of large amounts of RNA through sucrose-

density-gradient analysis (R. Brentani *et al.*, 1967).

Experimental

Preparative methods

Nuclear purification and fractionation. Nuclei were obtained from low-ionic-strength-buffered liver homogenates by low-speed centrifugation and were washed once with a mixture of detergents (Penman *et al.*, 1966). The resulting purified nuclei were fractionated into nucleoli and non-nucleolar nuclear fraction (called hereafter 'nucleoplasm') by a combined high-ionic-strength-enzymic extraction, and then by centrifugation through a 15–30% sucrose gradient (Penman *et al.*, 1968).

Preparation of ribosomes. Ribosomes were prepared by treatment of the post-mitochondrial supernatant with 5% (w/v) sodium deoxycholate in 0.03M-tris-HCl buffer, pH 8.2, and were collected by centrifugation at 105000g_{av.}, as reported by Brentani *et al.* (1968).

RNA extraction. RNA from the subcellular fractions was purified by phenol-detergent deproteinization, in the presence of bentonite at pH 7.4 and 4°C as reported by Brentani & Brentani (1969).

Other methods

Sucrose-density-gradient analysis. Gradients were performed as described by R. Brentani *et al.* (1967). All sucrose solutions contained 10mM-tris-HCl buffer, pH 7.4, 1mM-NaCl and 1mM-EDTA, and were pre-treated with diethyl pyrocarbonate (kindly supplied by Dr. E. Volkin) to inactivate endogenous ribonuclease (Solymosy *et al.*, 1968). RNA-containing fractions were pooled and the RNA was precipitated by the addition of MgCl₂, potassium acetate and ethanol to 1mM, 2.0M and 30% (v/v) final concentration respectively and was re-centrifuged on a second gradient.

The fractions under the 18S peak, referred to below as the '18S RNA fraction', both from nucleolar and nucleoplasmic RNAs, were pooled, precipitated as before and used for physico-chemical analyses.

Reaction with formaldehyde. The method described by Hall & Doty (1959) was adopted. RNA dissolved in 1mM-sodium phosphate buffer, pH 6.8, was treated with formaldehyde [final concentration 1% (v/v)] in quartz cells placed in a Cary 14 recording spectrophotometer equipped with a 0.1 slide-wire. The cell housing was connected to a water bath kept at 25°C. The wavelength was set at 270nm.

The reaction rates of the native and hydrolysed nucleolar 18S RNA fractions were compared. For this purpose, the RNA fraction was hydrolysed by incubation in 0.3M-KOH for 18h at 37°C. Then the

samples were neutralized with HClO₄, and KClO₄ was removed by centrifugation. Control experiments were performed with rRNA.

Ultraviolet absorbance-temperature profiles. These studies were done by Mandel & Marmur's (1968) procedure. RNA samples were dissolved in 5mM-sodium phosphate buffer, pH 7.4, containing 0.1M-NaCl and were placed in a stoppered quartz cell. Readings were performed with a PMQ II Zeiss spectrophotometer equipped with a cell holder that was heated by a thermostatically-controlled water bath. Temperature readings were made by inserting a thermometer in the reference cuvette. Corrections were made for water expansion and the cells were weighed at the start and finish of each experiment to correct for loss through evaporation.

Spectrophotometric titration. A Zeiss PMQ II spectrophotometer, fitted with a cell holder that was heated by a thermostatically controlled water bath, was used to measure extinctions. The pH of the solution was determined by a microelectrode (Metrohm A-CH 9100) used in conjunction with a Metrohm model E388 pH-meter. Acid or alkali additions were made with a Gilmont micropipette-burette, which delivered microlitre samples. After each addition, the cell contents were homogenized by bubbling with N₂ that had been passed through a pyrogallol solution.

Results

Density-gradient sedimentation analysis

RNA was fractionated with the aid of 5–20% sucrose gradients as outlined in the Experimental section. Fig. 1(a) shows clearly that, in nucleolar RNA, a prominent 18S peak exists, together with other RNA species, such as the 45S ribosomal RNA precursor, the heavy 28S RNA ribosomal RNA and a 4–7S shoulder that supposedly contains tRNA (Busch & Smetana, 1970).

On re-centrifugation on a second gradient (Fig. 1b), the 18S RNA fraction was purified yielding a small peak of contaminating 28S RNA, which was excluded from the experimental work by pooling only the appropriate gradient fractions.

Nucleoplasmic RNA is composed of the 28S and 18S fractions only (Fig. 1c). Re-centrifugation of the 18S peak results in considerable purification of the 18S nucleoplasmic RNA fraction (Fig. 1d).

Reaction with formaldehyde

The comparison of the slopes of the straight lines obtained by plotting observed hyperchromicity against time (Fig. 2) reveals that reaction with the native nucleolar 18S RNA fraction proceeds at a rate which is 88% of that observed for the hydrolysed 18S fraction. This reactivity can be taken as evidence

Fig. 1. Sucrose-density-gradient analysis of nuclear RNA

Nucleolar RNA (a) or nucleoplasmic RNA (c) were applied to 5–20% (w/v) sucrose gradients (see the Experimental section) and were centrifuged for 90 min at 40000 rev./min ($10500g_{av.}$) in the 50 rotor of a Spinco model L ultracentrifuge. After centrifugation, the bottom of the tubes were pierced and 40 fractions (15 drops) were collected. (b) and (d) Show the re-centrifugation of the nucleolar 18S RNA fraction and the nucleoplasmic 18S RNA fraction respectively. ■, represents the fractions pooled.

Fig. 2. Rate of reaction of nucleolar RNA with formaldehyde

Details are given in the Experimental section. Native RNA (●); hydrolysed RNA (○).

Fig. 3. Rate of reaction of ribosomal RNA with formaldehyde

Details are given in the Experimental section. Native RNA (●); hydrolysed RNA (○).

that this RNA presents a loose structure, stabilized by only 12% hydrogen bonding.

Control experiments with ribosomal RNA (Fig. 3) showed a reaction rate for the native polymer, which was 37% of that observed for hydrolysed rRNA.

This value agrees very well with that reported in the literature of 36% (Hall & Doty, 1959; Busch *et al.*, 1966).

Ultraviolet absorbance-temperature profile

Whereas the nucleolar 18S fraction displays only a slight hyperchromicity, which does not exceed 6% of the initial absorbance, nucleoplasmic RNA shows a 16% hyperchromicity, suggesting a structural organization closer to that of ribosomal RNA (Fig. 4). Taking the hyperchromicity of poly(A)·poly(U) as the 100% hydrogen-bonded standard (Bautz, 1963) gives only 12% hydrogen bonding for the nucleolar 18S RNA fraction, in agreement with the above results on reactivity towards formaldehyde.

Spectrophotometric titration

The change in E_{260} in the acid region of the pH scale, which reflects only modifications in the secondary structure of the molecule (Cox & Littauer, 1963), was determined (Fig. 5). The increment in absorbance caused by the addition of acid, as also observed for the effect of temperature, was not greater than 6% of the initial absorbance for the nucleolar 18S RNA fraction, indicating the relative absence of helical segments in this material. The nucleoplasmic 18S RNA fraction again exhibited a hyperchromicity of about 16%, related to its higher structural complexity.

Fig. 4. Ultraviolet absorbance-temperature profiles

Nucleolar 18S RNA fraction (○), nucleoplasmic 18S RNA fraction (●) or ribosomal RNA (◐), dissolved in 5 mM-sodium phosphate buffer, pH 7.4, containing 0.1 M-NaCl, was heated under the conditions described in the Experimental section.

Dissociation of uracil residues was measured by the examination of changes in the E_{230}/E_{260} ratio (Cox, 1966a,b) in the alkaline part of the pH scale (Fig. 6). A pK of 10.1 was observed for UMP residues in the 18S nucleolar RNA fraction. For ribosomal RNA, the pK was displaced towards a higher pH, as was expected for an organized polynucleotide.

Similarly, dissociation of guanine residues, also in the nucleolar 18S RNA fraction, was studied by examining the changes in the E_{270}/E_{260} ratio (Cox, 1966a,b) (Fig. 7). The observed pK was 10.05, which is in sharp contrast with rRNA where the pK is above 11.0.

The effect of temperature on the dissociation of cytosine residues, given by changes in the E_{280}/E_{260} ratio (Cox, 1966a) was studied by performing RNA titrations, as described in the Experimental section, at 25°, 45° and 75°C (Fig. 8).

It is clear that all the experimental points fit well along a single curve, with a pK of 4.10, which suggests that temperature has little effect on the dissociation of such residues.

The effect of ionic strength on the titration curve of base residues in nucleic acids can be measured spectrophotometrically (Cavalieri & Stone, 1955). Thus for *Escherichia coli* rRNA the pK in water is quite different from that obtained in 0.1 M-NaCl (Cox & Littauer, 1963).

Fig. 5. Dependence of E_{260} on pH

Nucleolar 18S RNA fraction (○), nucleoplasmic 18S RNA fraction (●) and ribosomal RNA (◐) were dissolved in 5 mM-sodium phosphate buffer, pH 7.4, containing 0.1 M-NaCl. For further details see the Experimental section.

Fig. 6. Dependence of the E_{230}/E_{260} ratio on pH

Nucleolar 18S RNA fraction (●) and ribosomal RNA (○) were dissolved in 5 mM-sodium phosphate buffer, pH 7.4, containing 0.1 M-NaCl. For further details see the Experimental section.

Fig. 8. Effect of temperature on the dissociation of cytosine

Nucleolar RNA was dissolved in 5 mM-sodium phosphate buffer, pH 7.4, containing 0.1 M-NaCl and titrated at different temperatures. ●, 25°C; ▲, 45°C; ■, 75°C. For further details see the Experimental section.

Fig. 7. Dependence of the E_{270}/E_{260} ratio on pH

Nucleolar 18S RNA fraction (●) and ribosomal RNA (○) were dissolved in 5 mM-sodium phosphate buffer, pH 7.4, containing 0.1 M-NaCl. For further details see the Experimental section.

Fig. 9. Effect of ionic strength on the dissociation of cytosine

Nucleolar 18S RNA fraction was dissolved in 5 mM-sodium phosphate buffer, pH 7.4, containing various concentrations of NaCl. ○, 10 mM-NaCl; ■, 60 mM-NaCl; ●, 0.1 M-NaCl. For further details see the Experimental section.

Fig. 10. Effect of ionic strength on the dissociation of cytosine

Nucleoplasmic 18S RNA fraction was dissolved in 5mM-sodium phosphate buffer, pH 7.4, containing various concentrations of NaCl. ●, 1 mM-NaCl; ○, 0.1 M-NaCl. For further details see the Experimental section.

The effect of ionic strength on the dissociation of cytosine residues was determined by titrating RNA solutions, which contained 10mM-, 60mM- and 0.1 M-NaCl (Figs. 9 and 10).

Again, for the nucleolar 18S RNA fraction, all experimental points fit a single curve, with a pK of 4.10, which shows that dissociation of cytosine is independent of the ionic strength, as is observed in polymers devoid of secondary structure, such as poly(U), poly(C) and oligo(A) (Michelson, 1963).

Titration of the nucleoplasmic 18S RNA fraction, on the other hand, shows a marked influence of the medium's ionic strength, since the pK of cytosine residues was shifted from 3.84 in 0.1 M-NaCl to 4.41 in 1.0mM-NaCl (Fig. 10), indicating that it is a highly organized polymer or family of polymers.

Discussion

The hyperchromicity observed for the nucleolar 18S RNA fraction is comparable with that obtained for RNAs known to be devoid of secondary structure, such as formaldehyde-treated tRNA (Boedtker, 1967) and poly(U) (Richards *et al.*, 1963; Inners & Felsenfeld, 1970). Further, this degree of hydrogen bonding is quite similar to that reported for haemoglobin mRNA, though it was obtained by different procedures (Hunt & Laycock, 1969).

The pK values for uracil and guanine residues in

the nucleolar 18S RNA fraction (Figs. 6 and 7) are close to 9.7, which is the pK for these residues in free uridylic acid and guanylic acid (Warner & Breslow, 1958). Further, the dissociation curves were similar to theoretical curves proposed for a structureless RNA, that is, the titration curve of a single group with pK 10.2 (Cox & Arnstein, 1963). In DNA and rRNA, however, the pK is higher than 11.0 because ionization of base residues is suppressed because of secondary structure (Cox, 1963).

The dissociation curves of both DNA and rRNA are markedly dependent on temperature (Cox, 1963). For the 18S nucleolar RNA fraction, the effect of temperature on the dissociation curve was studied only in the acid region of the pH scale, because of the low temperature coefficient of the pK s in the region (Cox, 1960) and also because the apparent heats of dissociation of keto groups are very high (Cox & Littauer, 1959).

The titration of the nucleolar 18S RNA fraction at different temperatures (Fig. 8) gave experimental points that fit well along a single curve, similar to that obtained for the titration of cytosine residues in an equivalent mixture of mononucleotides (Frederiq *et al.*, 1961), with a pK of 4.10, close to that of cytosine in cytidylic acid (4.2) (Peacocke, 1957) and in rRNA at 85°C (Cox, 1966b).

The comparison between the nucleolar and nucleoplasmic 18S RNA fractions shows them to present widely different physical structures, so that contamination of nucleolar RNA by material from elsewhere in the nucleus is hardly probable.

The examination of nucleolar RNA from HeLa cells (Penman *et al.*, 1968) reveals no 18S peak. In other tumours, such as the Walker adenocarcinoma (Jacob & Busch, 1967) or the AH 130 hepatoma (Akino & Amano, 1970), the 18S peak was present but was not as conspicuous as in our normal liver profiles (Fig. 1), a discrepancy that might have biological significance.

The stimulatory activity of nucleolar RNA prepared from nucleoli obtained by sonic oscillation of rat-liver nuclei was lower than that of nucleolar RNA extracted from nucleoli purified by salt or enzymic extraction of nuclei (M. Brentani *et al.*, 1967). Other workers (Busch & Smetana, 1970) have failed to reveal any such activity or any 18S RNA in their nucleolar preparations. However, sonic oscillation has been shown to damage microsomal mRNA severely (Sugano *et al.*, 1962; Ogata *et al.*, 1962; von der Decken & Campbell, 1964).

The gradients given in the present work and published elsewhere (Brentani & Brentani, 1969) tend to show that degradation seldom occurs when experiments are performed under carefully controlled conditions. If it is accepted that heterogeneous nuclear RNA is a precursor of several cytoplasmic RNA species, such as mRNA for instance, and also

that part of this RNA passes through the nucleolus on its way to the cytoplasm, it becomes quite possible that products of the processing of this heterodisperse RNA might accumulate in the nucleolus.

We have recently shown (Brentani *et al.*, 1972) that nucleolar RNA from chick embryos can stimulate the incorporation of glycine and proline, but not leucine, into a collagenase-soluble product, by rat-liver ribosomes under suitable conditions.

However, the fact remains that in the nucleolus an RNA species is accumulated, which, whatever its cellular origin or function, exhibits the structural features expected of an informational polyribonucleotide and unshared by its cytoplasmic or nucleoplasmic counterparts. Whether this material results from physiological processing of heterodisperse precursors or is accumulated by specific aggregation to nucleolar structures, possibly after cell disruption, is, we consider, beyond the scope of the present work.

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