Hybridization Properties of Nucleolar Ribonucleic Acid

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Rat liver nuclei were fractionated into chromatin and nucleolar fractions. Chromatin DNA, which does not form hybrids with rRNA, was, nevertheless, able to hybridize with ^{32}P -labelled total nucleolar RNA. The optimal temperature for this hybridization was 55°C when the reaction was carried out in $2\times\text{SSC}$ ($0.3\text{ M-NaCl}+0.03\text{ M-sodium citrate}$). The hybrids formed were specific, as judged by analysis of thermal elution profiles. The low T_m (73°C) observed could be explained by the low amount of DNA in the filters. The length of the hybridized sequences was estimated as 54 nucleotide pairs. Contamination of nucleolar RNA by nucleoplasmic RNA was ruled out by showing that the former was able to form more hybrids than the latter. Competition experiments showed that hybridization of nucleolar RNA, although not competed with by rRNA, suffered pronounced competition from total microsomal RNA, even though the levels of competition obtained did not equal those observed with cold nucleolar RNA as competitor.

For some years this laboratory has been concerned with the processing of mRNA in eukaryotes, and especially with the possible role of the nucleolus in this mechanism. This interest led to the demonstration that crude ribonucleoprotein fractions, of nucleolar origin, could stimulate the incorporation of labelled amino acids by mRNA-depleted ribosomes (Brentani *et al.*, 1964). It was next shown that purified nucleolar RNA was equally effective and more active than RNA obtained from other nuclear fractions (Brentani *et al.*, 1967). Fractionation of nucleolar RNA in sucrose gradients yields several fractions, of which the 18S RNA fraction is the only one that is active in the stimulation of amino acid incorporation by preincubated ribosomes (Brentani & Brentani, 1969). Similar results were reported for the AH130 hepatoma by Akino & Amano (1970). Work with chick embryo preparations has indicated that nucleolar RNA is able to direct the synthesis *in vitro* of collagen-like polypeptides by heterologous ribosomes (Brentani *et al.*, 1973).

The informational nature of the nucleolar 18S RNA fraction was also indicated by its structural simplicity (Brentani, 1971; Brentani *et al.*, 1972).

In all these experiments the possibility that the properties of nucleolar RNA were simply due to contamination of the nucleolar fraction by material from elsewhere in the nucleus has been excluded by comparison with the properties of nucleoplasmic RNA, which was much less efficient in both stimulation of amino acid incorporation and collagen

biosynthesis, and also exhibited much greater structural complexity.

As mentioned above, hybridization properties of nucleolar RNA, utilized to establish its informational nature (Brentani & Brentani, 1969), showed that it was able to react with DNA at concentrations in excess of those reported for rRNA in rat liver (Steele, 1968). To establish these results more firmly and to gain some insight into the role of the hybrid-forming nucleolar RNA, the present study was undertaken.

Materials and Methods

Materials

Triton X-100, Tween 40, 2,5-diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP), DNAase (deoxyribonuclease I) and RNAase (ribonuclease) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Pronase was obtained from Calbiochem, Los Angeles, Calif., U.S.A. $^{32}\text{P}_i$ (carrier-free) was obtained from the Instituto de Energia Atômica da Universidade de São Paulo, São Paulo, Brasil. All other chemicals were analytical grade and water for solutions was twice-glass-distilled.

Adult Wistar albino rats were obtained from a closed colony bred at this Institute.

Methods

Cellular fractionation. Liver nuclei were purified by methods using either Triton X-100 (Hymer & Kuff, 1964) or Tween 40 (Penman *et al.*, 1966) as

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detergents. To prepare the chromatin fraction, nuclei, purified by the method of Hymer & Kuff (1964), were extracted twice with 10 vol. of 0.05M-Tris-HCl buffer, pH 7.4, containing 0.14M-NaCl and 1mM-MgCl₂, and the final residue was suspended in 4 vol. of 0.05M-Tris-HCl buffer, pH 7.4, containing 2M-NaCl. After being stirred in the cold for 30 min the suspension was centrifuged at 40000g (r_{av} . 9cm) for 60 min. The supernatant was decanted and treated with 2 vol. of ethanol at 20°C. The precipitate obtained after centrifugation for 10 min at 10000g (r_{av} . = 10.5cm) was the chromatin fraction. To obtain labelled nuclear fractions by this method, rats were injected intravenously with 10mCi of ³²P_i 3h before being killed.

Ribosomes were recovered as the postmitochondrial supernatant by centrifugation at 105000g (r_{av} . = 6.4cm) for 90 min, after the addition of sodium deoxycholate (final concentration 0.5%); they were then preincubated for 20 min in a standard amino acid-incorporation mixture (Brentani *et al.*, 1968). Pre-incubated ribosomes were recovered as the pellet obtained by centrifugation of the incubation mixture for 120 min at 105000g. A microsomal fraction was also obtained by centrifugation of the postmitochondrial supernatant at 105000g for 60 min.

Extraction of nucleic acids. DNA was purified from the chromatin fraction obtained from Triton-extracted nuclei, by the method of Church & McCarthy (1967). Purified DNA was dissolved in 0.1×SSC* and treated with pre-boiled RNAase (50 µg/ml) at 37°C. After 30 min, self-digested Pronase (50 µg/ml) was added and the incubation continued for another hour. The solution was deproteinized with chloroform-octanol as before and the DNA precipitated by addition of 2 vol. of ice-cold ethanol.

RNA was purified from ribosomes, microsomal fraction, nucleoli and nucleoplasmic fractions by methods previously described (Brentani & Brentani, 1969).

RNA-DNA hybridization. DNA from the chromatin fraction, obtained from Triton-extracted nuclei, was denatured with 1M-NaOH for 10 min. Alkaline DNA solutions were neutralized, brought to 2×SSC, and applied to wet Millipore GS filters, with moderate suction. Between 25 and 35% hyperchromicity was caused by alkali denaturation. Hybrids were formed by immersing DNA-containing filters in RNA solutions (2×SSC as solvent). The hybridization reaction was allowed to continue for 17h at 55°C, unless otherwise specified. Incubation was stopped by dilution in ice-cold 2×SSC.

* Abbreviation: SSC, 0.15M-NaCl + 0.015M-sodium citrate.

After being washed three times with cold 2×SSC, filters were placed in 2×SSC containing RNAase (30 µg/ml) at 37°C for 30 min, and then once more in 2×SSC alone. After the filters had been dried in an oven, radioactivity was determined in a Beckman LS-100 liquid-scintillation spectrometer, in PPO (4g/l)-POPOP (100mg/l)-toluene as scintillator.

After the radioactivity of each filter had been determined, the filters were washed with chloroform, dried and incubated in HCl at 98°C for 20 min; the amount of DNA retained by each filter was determined, by reading the absorbance of the solution at 268 nm (27.8 units of absorbance corresponding to 1 mg of DNA/ml).

For determination of thermal elution profiles, filters were incubated in 2×SSC for 10 min at increasing temperatures. After equilibration at each temperature, a sample of the solvent was removed and counted for released radioactivity.

Results and Discussion

Even though the relatively low specific activity of the RNA species used in the present experiments would preclude the detection of rRNA-DNA hybrids, the DNA used was purified by a procedure which yields a 'chromatin DNA fraction' which was previously found to be unable to form hybrids with specifically labelled rRNA (Brentani *et al.*, 1973).

Despite this, as shown by Brentani & Brentani (1969) and in the present experiments, nucleolar RNA appears to contain RNA species that are able to form hybrids with this purified 'chromatin DNA' and which are presumably different from rRNA or its precursors.

Studies on the kinetics of reassociation of denatured DNA show that the reassociation rate is dependent on several factors, such as salt concentration, solvent viscosity and base composition (Thrower & Peacocke, 1966). Moreover, the optimal temperature of reassociation is related to the 'melting' temperature (T_m). Likewise, for RNA-DNA hybridization the optimal temperature is proportional to the base composition of the hybridizing RNA. Thus mRNA species react at lower temperatures than rRNA species (Huystee & Cherry, 1966; Gillespie, 1968). Fig. 1 shows that hybridization between nucleolar RNA and chromatin DNA reaches a maximal value at 55°C.

Since lowering the reaction temperature favours formation of mismatched, thus unspecific, hybrids (Walker & McLaren, 1965), it is important to show that hybrids formed between nucleolar RNA and chromatin DNA are specific. It is known that the thermal stability of a nucleic acid duplex is extremely sensitive to the presence of mismatched pairs within the polynucleotide strands (Bautz &

Fig. 1. Influence of temperature on hybridization between nucleolar RNA and chromatin DNA

Millipore GS filters, containing 5 µg of chromatin DNA/filter, were immersed in ^{32}P -labelled nucleolar RNA, prepared as described under 'Methods', dissolved in $2\times\text{SSC}$ and incubated at various temperatures for 17h. The filters were then washed and their radioactivity determined as described under 'Methods'.

Bautz, 1964; Kotaka & Baldwin, 1964). Thermal stability of hybrids between nucleolar RNA and chromatin DNA was studied by analysis of their thermal elution profiles (Fig. 2). The RNA loss from our hybrids followed a very steep curve, in which most of the transition (80%) from double- to single-stranded forms occurred in a narrow (15°C) interval, suggesting homogeneity of the formed duplexes.

The observed T_m (73°C) is rather low, but this can be explained by the low amount of DNA present in the filters. In *Escherichia coli* the T_m of hybrids of pulse-labelled RNA shifts from 87°C to 79°C when filter-bound DNA is changed from 100 to 1 µg (Kennell, 1971).

Duplex stability is also strongly dependent on the size of the matched polynucleotide sequences. Depending on the G+C content, the minimal size for a stable complex is 10–20 nucleotides. The stability of a complementary duplex of 25–50 nucleotides approaches that of much longer complexes (Niyogi, 1969). By integrating the expression (Kennell, 1971):

$$\frac{d \ln S}{dT} = \frac{\Delta H}{R \cdot T^2} \quad (1)$$

where S is the equilibrium constant for the process of increasing the length of a sequence of bonded base pairs to the extent of one base pair at the expense of the adjoining sequence of unbonded base pairs; ΔH is the heat of forming a bonded base pair from

Fig. 2. Thermal stability of hybrids between nucleolar RNA and chromatin DNA

Hybrids were formed at 55°C as described under 'Methods'. After enzymic digestion, filters were incubated in $2\times\text{SSC}$ for 10min at 50°C. After removal of a sample for determination of released radioactivity, filters were next incubated for a further 10min at 55°C and so on until the temperature had risen to 85°C. The cumulative released radioactivity was plotted as a percentage of the radioactivity on the unincubated filter.

an unbonded base pair and is assumed to be -7kcal/mol of base pair (Kennell, 1971). Between 73°C (our experimentally determined T_m) and 55°C (the optimal reaction temperature) we obtain a value of S of 1.81 at 55°C. This value can be substituted into the equation:

$$K = t \cdot S^n \quad (2)$$

where K , the association constant of the hybrid DNA–RNA complex, is in the neighbourhood of 10^{10} (Kennell, 1971) and t , the equilibrium constant for the 'nucleation' event, is equal to 10^{-4} (Crothers, 1969). This calculation leads to an estimate of $n = 54$ base pairs.

For the interpretation of hybridization reactions involving non-ribosomal nucleolar RNA, it is imperative to show that the experimental results are not a consequence of nucleolar contamination, during cell fractionation, by material from elsewhere in the nucleus. This is indicated by the fact that the method used for nucleolar purification effects a complete separation of nucleoli and heterogeneous nuclear RNA (Penman *et al.*, 1968; Vesco & Penman, 1968).

As shown in Fig. 3(a), nucleolar RNA is much more efficient than nucleoplasmic RNA in the formation of hybrids with chromatin DNA. Curves like those shown in Fig. 3(a) can be redrawn according to the formula

$$\frac{1}{H_y} = \frac{1}{D} + \frac{K}{D\alpha} \cdot \frac{1}{R} \quad (3)$$

Fig. 3. (a) Comparison of the efficiency of nucleolar and nucleoplasmic RNA in forming hybrids with chromatin DNA and (b) reciprocal-plot hybridization curves between chromatin DNA and nucleolar and nucleoplasmic RNA

There was $5\mu\text{g}$ of DNA/filter. ●, Nucleolar RNA (specific radioactivity, 449 c.p.m./ μg); ■, nucleolar RNA (specific radioactivity, 297 c.p.m./ μg); ○, nucleoplasmic RNA (specific radioactivity, 267 c.p.m./ μg); □, nucleoplasmic RNA (specific radioactivity, 75 c.p.m./ μg).

Fig. 4. Competition between labelled nucleolar RNA and unlabelled RNA of various types

(a) shows the competition between labelled nucleolar RNA and unlabelled nucleolar RNA (●), unlabelled microsomal RNA (▲) and unlabelled ribosomal RNA (■); $150\mu\text{g}$ of labelled nucleolar RNA (specific radioactivity, 449 c.p.m./ μg) was hybridized to filters containing $5\mu\text{g}$ of chromatin DNA/filter, in the presence of increasing amounts of the various unlabelled species of RNA. (b) shows a plot of the competition experiments according to the equation

$$\frac{1}{H_r} = 1 + \beta Z$$

The terms are defined in the text and the symbols are the same as for (a).

where H_r is the radioactivity in the hybrid (c.p.m.); R is the concentration of total RNA; D is the concentration of DNA; K is the dissociation constant of the hybrid DNA-RNA complex; α is the fraction of total RNA present as messenger (Lavallé & de Hauwer, 1968). The intercept on the ordinate of a reciprocal plot is used to determine precisely the radioactivity in the hybrid (c.p.m.) at saturation. For different experiments this value is

normalized so that, in reciprocal plots like Fig. 3(b), the various curves can be directly compared.

Some insight into the role of the hybridizing RNA sequences can be gained by performing hybridization of nucleolar RNA in the presence of increasing concentrations of microsomal RNA. Significant competition was observed (curve 2, Fig. 4a). When RNA, obtained from ribosomes that have been depleted of endogenous mRNA by preincubation, is

used as competitor (curve 1, Fig. 4a), very little competition occurs. Competition with microsomal RNA is, however, less efficient than that obtained with nucleolar RNA (curve 3, Fig. 4a), indicating that some, but not all, nucleolar RNA sequences are shared by cytoplasmic non-ribosomal RNA. The results of the competition experiments can also be redrawn according to the formula:

$$\frac{1}{\bar{H}_y} = 1 + \beta Z \quad (4)$$

where $\bar{H}_y$ = amount of radioactivity in the hybrid (c.p.m.) divided by the amount of radioactivity in the hybrid (c.p.m.) when there is no competing RNA. Let Z be the concentration of competing RNA and R the concentration of total RNA [as defined in eqn. (3)], then Z is Z/R , which is the amount of competing RNA added, expressed as the amount of labelled RNA used. β is the ratio of the mRNA content in the two RNA preparations (Lavallé & de Hauwer, 1968).

As shown in Fig. 4(b), the experimental data for the dilution with homologous unlabelled RNA fitted well with the theoretical line having a slope of one (curve 1, Fig. 4b). The second control curve (curve 3, Fig. 4b) shows very slight interference by the rRNA preparation which is supposed to be devoid of mRNA. The slope of the straight line obtained in the competition experiment between labelled nucleolar RNA and unlabelled microsomal RNA indicates that the latter shares 30% of the hybrid-forming nucleolar RNA sequences.

It has already been suggested that the nucleolus might be involved in the processing of mRNA (Brentani, 1968). Experimental support for this hypothesis was given by the demonstration that nucleolar RNA could code for the synthesis *in vitro* of a specific cytoplasmic protein, namely collagen (Brentani *et al.*, 1973).

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