SECONDARY STRUCTURES FORMED BY RANDOM RNA SEQUENCES

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SUMMARY: Three techniques (optical melting curves, sedimentation in the presence and absence of dimethylsulfoxide, and resistance to RNase T1) are used to investigate different aspects of the structure of random RNA sequences. The hypochromicity of melting curves indicate that these sequences have about 60% base-pairing, but the low T_m and large σ_T suggest that the base-pairing is much less stable than that observed in rRNA. Both the small hydrodynamic radius revealed in sedimentation studies and the relatively low resistance to RNase are interpreted as confirming the presence of base-pairing but base-pairing which is disorderly.

Previous studies have suggested that T4-specific mRNA forms stable secondary structures (1), like most sequenced RNAs (tRNA, rRNA, RNA of the RNA bacteriophages). Many workers have postulated that such structures are biologically important but it has also been suggested that a certain amount of base-pairing might be inevitable even in a random RNA sequence. This possibility has most recently been investigated theoretically by Gralla and DeLisi who studied random sequences generated by computer (2). These sequences were shown to average about 50% base-pairing; however, the evaluation of the stability of the proposed structures depends upon thermodynamic considerations which are only imperfectly understood (3-5). Moreover, no attempt was made to estimate whether such hypothetical random sequence structures could have the high melting temperatures and sharp (cooperative) melting curves which are observed with natural RNAs. It therefore seemed to us worthwhile to obtain experimental data on the secondary structure of random sequences in order to answer these questions directly. To do this, we have studied the so-called "random copolymer"

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RNA sequences synthesized with polynucleotide phosphorylase.[1]

MATERIALS AND METHODS

Random copolymer (Miles) contained ACGU in the following proportions: 1.1, 1.2, 1.0, 1.3 and was reported to have a minimum molecular weight greater than 100,000. It was repurified by chromatography on a Biogel P60 column. All experiments were done with the material which eluted in the void volume. As shown in Figure 2a, this material sediments as a symmetric peak at about 4.1S.

RESULTS AND DISCUSSION

A direct measure of the amount of base-pairing can be obtained from optical melting curves (6). The shape of such melting curves and the T_m give us additional information about the stability of the base-paired regions. Optical melting curves of random copolymer and a 16S rRNA (E. coli) control are presented in Figure 1. The hypochromicities indicate a considerable amount of base-pairing in both cases (60% for the random copolymer, 70% for 16S rRNA). The 16S rRNA is more highly base-paired than random copolymer, but an even more important difference is indicated by the shape of the melting curves. The rRNA control shows an S-shaped melting curve with the high cooperativity which has been regarded by others as typical of structures with large amounts of highly ordered base-pairing (7). The random copolymer, on the other hand, melts over a much wider range of temperatures with no S-shape as we would expect of a randomly paired structure with disordered base-pairing. The quantitative measure of cooperativity σ_T is much larger for the random copolymer than for the rRNA (low σ_T indicates a sharper melting curve) (8).

The T_m of random copolymer is also 11° lower than that for rRNA. From the data published by Spirin (9), we predict a 5° decrease in T_m for every 10% decrease in (G+C) if the secondary configurations are similar. But this would only account for 2 or 3° of the 11° difference in T_m [random copolymer

[1] We will assume that these sequences are truly random even though it is remotely possible that the enzyme could synthesize predominantly double-stranded sequences if base-pairing were permitted between incoming nucleoside diphosphate substrates and already synthesized single-stranded regions.

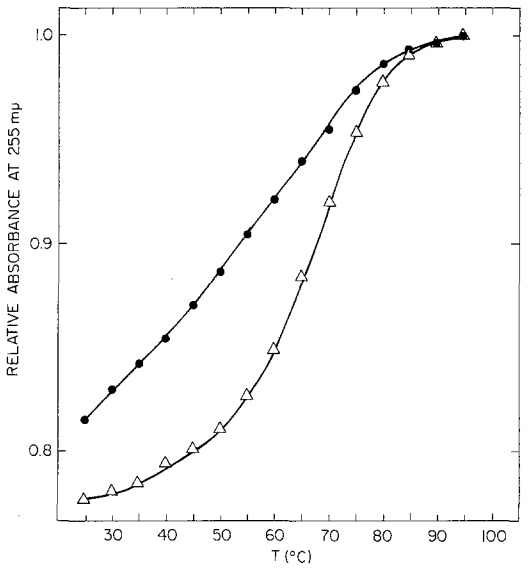

Fig. 1. Optical melting curves of rRNA and random copolymer. 0.6-0.7 absorbance units of rRNA or random copolymer degassed in 1 ml of 0.3 M NaCl, 0.001 M $MgCl_2$, 0.01 M Tris-Cl, pH 7.3 were placed in teflon-stoppered quartz cuvettes of 1-cm path length and the absorbance at 255 nm determined as a function of temperature. Readings have been corrected for solvent expansion. Hypochromicity $(1-[A_{25°C}/A_{85°C}]_{255nm})$ (6), T_m and cooperativity of melting $\sigma_T(T[\bar{h} + 0.192\ (hmax-1)] - T[\bar{h}-0.192\ (hmax-1)]$, where $\bar{h} = 1/2\ (hmax +1)$ and $hmax = \frac{A \text{ at } 95°C}{A \text{ at } 25°C}$ (8) were as follows:

●——● Random Copolymer ($h = 0.18$, $T_m = 55°C$, $\sigma_T = 20°C$)

△——△ 16S rRNA ($h = 0.21$, $T_m = 66°C$, $\sigma_T = 13°C$)

48% (G+C) versus E. coli rRNA, 52.5% (G+C) (10)]. Thus the low T_m, like the low cooperativity, suggests a greater number of base-pairing imperfections.

In order to learn more about the native conformation of the random copolymer, we have centrifuged this material in sucrose gradients in the presence and absence of dimethylsulfoxide (DMSO), a solvent which causes complete denaturation (11) (Figure 2). Fraction 5 in Figure 2a, consisting of material which sedimented at the same position as tRNA on an aqueous sucrose gradient, sedimented slower than tRNA when centrifuged again under denaturing conditions (Figure 2b). Since the sedimentation coefficient in DMSO gives us a measure of the molecular weight which is unaffected by conformation, this implies that the 4S random copolymer is smaller than tRNA,

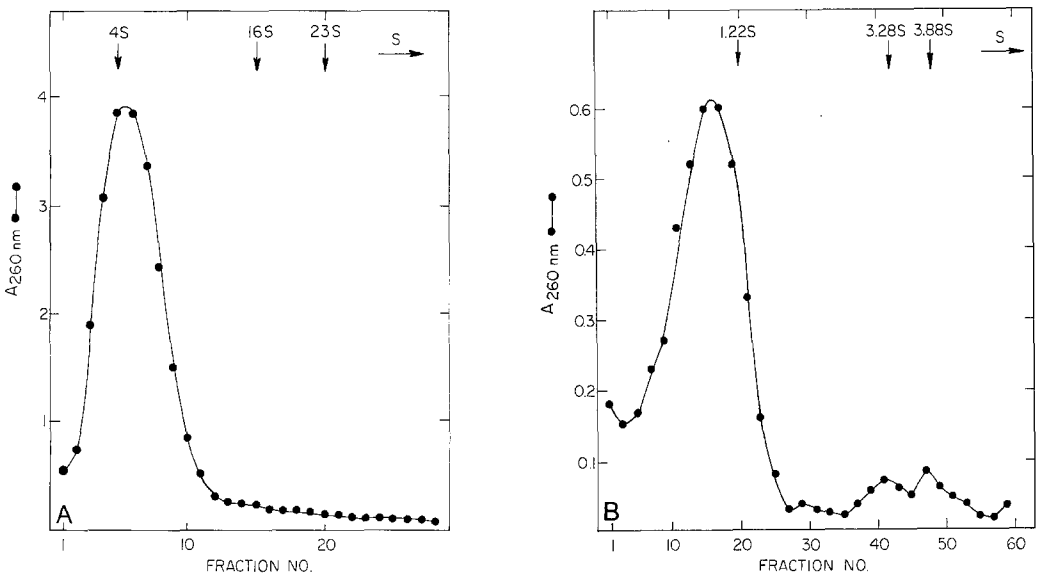

Fig.2. Sedimentation of random copolymer in the absence (a) or presence (b) of DMSO. About 30 absorbance units of random copolymer were layered onto a 5-20% sucrose gradient in 0.1 M NH_4Cl, 0.01 M NH_4 acetate, pH 4.6, and centrifuged in an SW27 rotor at 24,000 rpm for 21.7 h. The RNA was precipitated from the fractions collected by adding 0.1 vol 3M Na acetate, pH 5.1 and 2 vol ethanol at -20°C. b) 2.4 absorbance units from fraction 5 (a), which sedimented at the position of a tRNA marker, were mixed with 100 ul of dimethylformamide and layered onto a 5 ml linear DMSO gradient. The gradient was established between 2.5 ml of d_6-DMSO (containing 0.25 g RNase-free sucrose and 1 mM EDTA, pH 7.0) and 2.65 ml of d_6-DMSO:DMSO (1:9) containing 1 mM EDTA, pH 7.0. Centrifugation was in an SW65 rotor at 64,000 rpm at 27°C for 12 h. Six drop fractions were collected into 0.5 ml of sterile, doubly distilled water and the RNA was precipitated as in (a). The ultraviolet absorbance of each fraction was determined.

The arrows labeled 1.22S, 3.28S and 3.88S indicate the positions of sedimentation and the $S_{25,DMSO}$ of ^3H-labeled 4S, 16S, and 23S E. coli stable RNA species used as markers.

averaging 65 nucleotides in length. In order to cosediment with tRNA in an aqueous sucrose gradient, it must therefore have a smaller hydrodynamic radius than tRNA.

Transfer RNA, with a high degree of ordered base-pairing, has been shown by x-ray crystallography (12) to have an elongated L-shaped structure. A perfectly base-paired hairpin loop would have an even larger hydrodynamic radius while we would expect random copolymer to have a smaller hydrodynamic radius than tRNA if it were highly base-paired in a disordered fashion.

If random copolymer were characterized by disordered base-pairing, one

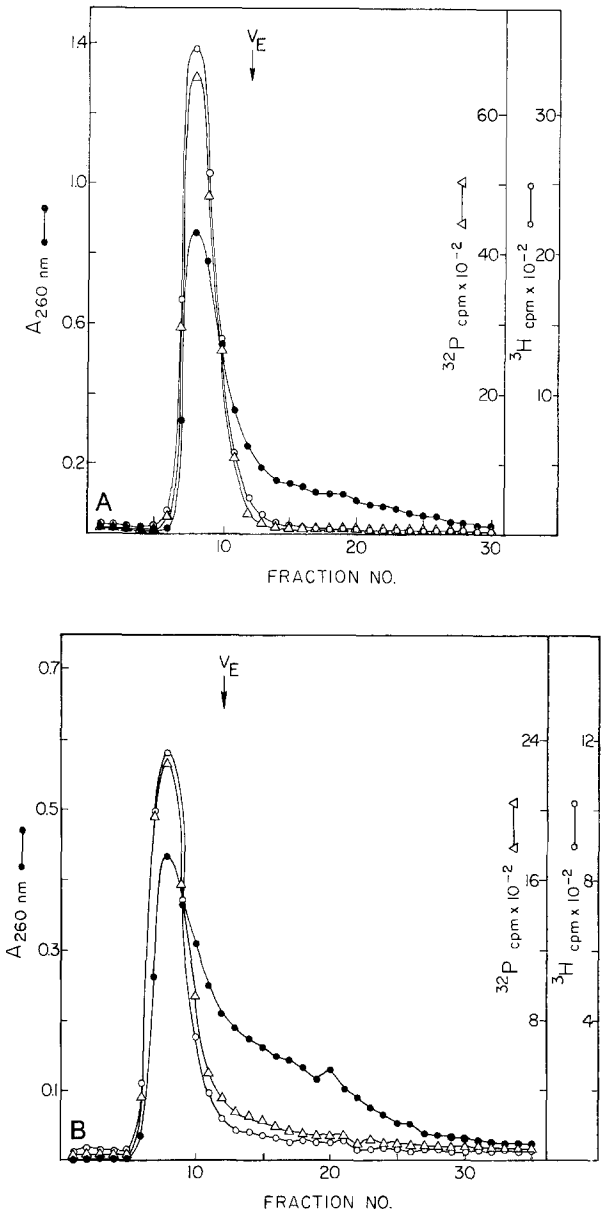

Fig. 3. RNase T1 digestion of random copolymer (prepurified on a Biogel P60 column and 4.1S in size), ^{32}P-labeled T4-specific mRNA and ^{3}H-labeled Escherichia coli rRNA at enzyme to substrate ratios of 1:14400 (a) and 1:1600 (b). 0.24 mg of RNA were treated with RNase T1 in 0.2 M NaCl, 0.02 M Mg acetate, 0.05 M Tris-Cl, pH 7.5 in the presence of bovine serum albumin added to give a final concentration of 0.05 mg/ml at 4°C for 16 h. The digested RNAs were eluted from a Biogel P60 column with 1 M NaCl, 0.05 M Tris-Cl, pH 7.7, 0.001 M EDTA. Fractions were collected and the ultraviolet-absorbance was determined. The arrow V_E marks the cut-off point used in computing the % of material running in the included versus the excluded volumes.

would expect the helical regions to be destabilized and therefore to be more susceptible to RNase T1 digestion. This is confirmed by studies comparing the RNase T1 digestion rates of the 4.1S random copolymer with those of ^{3}H-labeled stable RNA and ^{32}P-labeled T4-specific mRNA (Figure 3). At an enzyme to substrate ratio of 1/14400, 37.5% of the random copolymer has been digested to fragments small enough to appear in the included volume of the Biogel columns. Only 4.3% of the stable RNA and 2.1% of the T4-specific mRNA appear in the included volume at this stage of the digestion.

Previous experiments show that stable RNA as well as T4-specific mRNA are very quickly degraded to fragments in the size range 30 to 200 nucleotides which are only slowly digested further to give smaller fragments which would run in the included volume of the Biogel P60 column. (From careful sizing of the various fractions in the included volume, using the method of Furlong (13), we conclude that the fragments in the included volume are 20 nucleotides or less in size.) From this we may be certain that the difference in the apparent digestion rates of random copolymer and the natural RNAs is not due to their large initial difference in size.

When the enzyme to substrate ratio is increased nine-fold to 1/1600 (Figure 3b), 49% of the random copolymer appears in the included volume as compared to only 25% of the stable RNA and 17% of the T4-specific mRNA. Thus a nine fold increase in nuclease levels is not suffiecient to increase the digestion of the rRNA or T4 mRNA to the 37.5% level observed with random copolymer at the lower enzyme concentration.

This large difference in sensitivities was observed when the digestion was carried out at 4°C. Inspection of Figure 1 suggests that there might be even greater effects at 37°C where a substantial part of the random copolymer secondary structure has already begun to melt. This rate difference cannot be ascribed to different base composition. The random copolymer used here has 21.7% G while the T4 mRNA has 25% G (14) and E. coli rRNA has 29.5% G (10). Since RNase T1 is specific for G residues, one might have

expected the random copolymer to be digested more slowly, the opposite of the actual results.

Could the RNase T1 digestion results be interpreted to mean that there is much less total base-pairing in the random copolymer than in ribosomal RNA or T4 mRNA? Experiments with model compounds suggest that a truly single-stranded molecule should be digested even more rapidly than random copolymer; and the results presented in Figure 1 indicate that in fact there is a high percent base-pairing in random copolymer. More likely the greater resistance of the ribosomal RNA and T4 mRNA to RNase T1 indicates the presence of long runs of accurate base-pairing which have arisen because of evolutionary selection pressures which we do not yet understand. The lack of cooperativity in the melting curves of random copolymer suggests disordered base-pairing which should result in the greater nuclease sensitivity which we in fact observe.

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