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Comparative Optical Property Studies on Polycistronic R17 Phage Ribonucleic Acid and Rabbit Globin Messenger Ribonucleic Acid†

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ABSTRACT: Optical density and circular dichroism (CD) properties of the polycistronic R17 phage RNA and rabbit globin mRNA have been compared in the presence and absence of Mg^{2+}. If Mg^{2+} is present, the CD melting profile is more complex for R17 RNA than for the eukaryotic messenger, whereas the main positive CD band at about 266 nm is similar for both RNAs at 28°. In the absence of Mg^{2+} and in a buffer of low ionic strength the melting profiles are similar for R17 RNA and rabbit globin mRNA. However, the mRNAs of the two different sources show now a pronounced difference in their CD spectra at 28°. Calculations of CD curves have been performed which show that the observed CD differences are due to stacked adenylate segments of an average length of 40–60 nucleotides, present in the rabbit globin mRNA and absent in the R17 RNA.

Most physical studies on the conformational properties of ribonucleic acids in solution have so far been carried out on synthetic polyribonucleotides, transfer, ribosomal, and viral RNAs. It seemed worthy to us to compare conformational properties of rabbit globin mRNA with those of the R17 polycistronic mRNA. In the first case one is dealing with a messenger which is covalently bound to polyadenylic sequences, whereas no such sequences seem to occur in the R17 RNA.

Much progress has been made in determining nucleotide sequences in the R17 phage RNA (Adams *et al.*, 1972a,b; Cory *et al.*, 1972). Sequencing results at the 5′- and 3′-ends of R17 RNA suggest the presence of tightly hydrogen-bonded hairpin loops at both ends (Adams *et al.*, 1972a; Cory *et al.*, 1972). Besides the existence of hairpin loops there is reason to believe that R17 RNA has a defined tertiary structure (Fukami and Imahori, 1971; Jeppesen *et al.*, 1970; Phillips and Bobst, 1972). The sequencing work of rabbit mRNA is still in its early beginnings. Lim and Canellakis (1970) isolated from rabbit mRNA chain lengths of 50–70 nucleotides, containing 70% AMP. Burr and Lingrel (1971) and Hunt (1973) identified polyadenylate sequences at the 3′-termini of globin mRNA. The sequences appear to be homogeneous and of approximately the same length for both α- and β-globin mRNA (Hunt, 1973). A polyadenylate region of about 50 nucleotides was found in mouse globin mRNA (Morrison *et al.*, 1973). The influence of Mg^{2+} on both mRNAs was examined in view of its importance to the conformation and function of RNAs. It has been shown for certain tRNAs by circular dichroism (CD) that a conformational change takes place upon the addition of Mg^{2+} (Willick and Kay, 1971). With respect to the biological role of Mg^{2+} in the case of polycistronic mRNA f_2, it was found that Mg^{2+} strongly influences the kind of final products formed under the direction of phage f_2 RNA (Zagorski *et al.*, 1972). Significant changes in the incorporation of some amino acids into protein were also seen with R17 RNA, when it was first incubated in the presence of Mg^{2+} before being used as messenger (Fukami and Imahori, 1971).

We report here the results of a comparative optical property study on viral and eukaryotic mRNAs with and without the addition of Mg^{2+}. Although one is far from understanding the exact nature of secondary and tertiary structures for systems as complex as those studied here, the two types of messengers show significant differences with respect to their spectroscopic and thermodynamic properties.

Materials and Methods

R17 RNA. Escherichia coli K12 Hfr RNase $^{-}D_{10}$ was grown in defined medium (Gesteland and Boedtker, 1964) to a cell density of 4×10^8/ml and then infected with R17 at a multiplicity of 10. The phage was purified and its RNA isolated according to the procedure of Gesteland and Spahr (1970). The absence of hidden breaks was verified by sedimenting fractions through a sucrose density gradient containing formaldehyde (Boedtker, 1968).

The rabbit globin mRNA (a mixture, coding for the α- and β-globin chains) was a gift from J. B. Lingrel. It had been prepared according to procedures described previously (Evans and Lingrel, 1969; Lingrel *et al.*, 1971). The degree of contamination of the rabbit globin mRNA with RNA containing no or small poly(A) stretches was determined through oligo(dT) cellulose affinity chromatography; 20% or less of the material used for this study did not bind to the column

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TABLE I: Percentage of Bases in Base-Paired and Non-Base-Paired Regions of R17 RNA and Hb mRNA.[a]

	R17 RNA			Hb mRNA		
Base	Base Paired (%)	Nonbase Paired (%)	Total (%)	Base Paired (%)	Nonbase Paired (%)	Total (%)
A	15	8	23[b]	14	15	29[c]
U	15	11	26[b]	14	5	19.0[c]
G	17.5	8.5	26[b]	20.5	4.5	25[c]
C	17.5	7.5	25[b]	20.5	6.5	27[c]
AU	30	19	49	28	20	48
GC	35	16	51	41	11	52
AUGC	65	35	100	69	31	100

[a] Calculated according to Gould and Simpkins (1969) for both RNAs at 28° in 0.01 M Tris–0.01 M NaCl–0.001 M EDTA (pH 7.5). [b] Taken from Mitra *et al.* (1963). [c] Taken from Williamson *et al.* (1971).

(J. B. Lingrel and M. R. Morrison, private communication). It is also likely that the purified α- and β-globin mRNAs show some heterogeneity with respect to the length of the poly(A). For that reason the spectroscopic data will only reflect an average length of the homopolymer present in rabbit mRNA.

Concentration Determination. Concentrations of the RNA solutions were determined from A_{258} nm. The value for the molecular residue extinction coefficient was obtained on the basis of phosphorus analysis (Ames and Dubin, 1960). The ϵ values at 258 nm (per M nucleotide residue per cm) in 0.001 M EDTA–0.01 M NaCl–0.01 M Tris-HCl buffer (pH 7.5) are $7.5 \times 10^3 \pm 1\%$ and $7.9 \times 10^3 \pm 1\%$ for R17 RNA and rabbit globin mRNA, respectively.

Circular Dichroism. CD spectra were obtained on a Cary Model 6002 CD attachment to a Cary 60 recording spectropolarimeter, equipped with a thermostatable cylindrical cell holder. The curves were run at 28° unless otherwise stated. Results are reported in terms of mean residue molecular ellipticity, $[\theta]$, and the units of $[\theta]$ are (deg cm^2)/dmol. Measurements were usually made in 10-mm cells at a nucleotide residue concentration of about 0.1 mM. However, for solutions containing EDTA 0.2–0.3 mM solutions and 5-mm cells were used.

For the CD variable-temperature experiments water from two Haake thermostats, coupled with a water bath cooler, was circulated through a thermostatable cylindrical cell holder. One Haake bath was used in order to keep the temperature at about 26° in the cell compartment, whereas with the second bath the cell was equilibrated to the desired temperature. The temperature was increased at a rate of 2–3° at a time, and 15–20 min were allowed for equilibration, before the CD spectra were recorded between 310 and 250 nm. For the temperature measurements a sensitive thermistor was used which was inserted through a Teflon stopper into the top of the cell. During each scanning the temperature was monitored at 310, 266, and 250 nm. No significant temperature change was observed within this wavelength range. No correction for thermal expansion of the solution was made.

Temperature–Absorbance Profiles. The absorbance *vs.* temperature profiles were obtained from the Beckman Kintrac VII spectrophotometer equipped with a thermostated cell housing. To minimize temperature gradients the solutions were continuously mixed with a built-in magnetic stirrer. The temperature was monitored by a thermistor probe inserted in the cell containing the polynucleotide solution. The readings were corrected for the expansion of water upon heating.

Chemicals. Buffer components and other chemicals were reagent grade. The solutions were prepared with deionized and freshly glass-distilled water.

CD Data Analysis. The CD of globin mRNA and R17 RNA was divided into two parts. The first one gives rise to a similar CD contribution for both RNAs, whereas the second part shows a different contribution, reflecting the difference in sequential arrangement of the adenylic acid residues at the 3′-end. It is reasonable to assume that the CD contribution of the first part is similar for both RNAs since their overall base composition does not differ much (*cf.* Table I). Equation 1 was used to calculate the CD spectrum shown in Figure 2a. The best fit of the calculated spectrum with that of R17 RNA was obtained by varying F. The parameter F reflects the fraction of nucleic acid residues which form a homopolymer consisting of adenylic acid building blocks. The addition and subtraction of a fraction of the two hypothetical CD curves, $[\theta_1]$ and $[\theta_2]$, from the rabbit globin mRNA CD spectrum, $[\theta$ globin], was done with a Du Pont 310 curve resolver.

$$[\theta_{\mathrm{R17RNA}}(\lambda)] \simeq [\theta_{\mathrm{calcd}}(\lambda)] = [\theta_{\mathrm{globin}}(\lambda)] - 2\mathrm{F}[\theta_1(\lambda)] + 2\mathrm{F}[\theta_2(\lambda)] \quad (1)$$

The CD curves for the hypothetical polyribonucleotides were calculated according to eq 2 and 3. These equations were derived from the general equation for calculating optical properties of polynucleotides based on the nearest neighbor scheme (Cantor *et al.*, 1966). In eq 3 contributions from nearest neighbor interactions of adenylic acid with the other three bases only were considered. End effects have been ignored. $[\theta_{\mathrm{A}j}]$ and $[\theta_{i\mathrm{A}}]$ are experimental mean residue molecular ellipticities of the dimers ApJ and IpA. For $[\theta_i]$ the mean residue molecular rotation of the nucleotide I was used. The experimental values were taken from Warshaw and Cantor (1970). The mole fraction of dimer and monomer is f. For a given wavelength

$$[\theta_1] = [\theta \text{ poly(C-U-G)}_n\text{-Am)}] = {}^1/_2[\theta \text{ poly(A)}] + {}^1/_2\left\{2\sum_{\substack{i=1\\ i\neq j\\ i\neq \mathrm{A}}}^{3}\sum_{j=1}^{3} {}^1/_6[\theta_{ij}] - \sum_{i=1}^{3} {}^1/_3[\theta_i]\right\} \quad (2)$$

$$[\theta_2] = [\theta \text{ poly(A-C-A-U-A-G)}] = 2\sum_{\substack{j=1\\ j\neq \mathrm{A}}}^{3} 2f_{\mathrm{A}}f_j[\theta_{\mathrm{A}j}] + 2\sum_{\substack{i=1\\ i\neq \mathrm{A}}}^{3} 2f_i f_{\mathrm{A}}[\theta_{i\mathrm{A}}] - \sum_{i=1}^{4} f_i[\theta_i] \quad (3)$$

$$f_{\mathrm{U}} = 0.5; f_{\mathrm{C}} = 0.166; f_{\mathrm{U}} = 0.166; f_{\mathrm{G}} = 0.166.$$

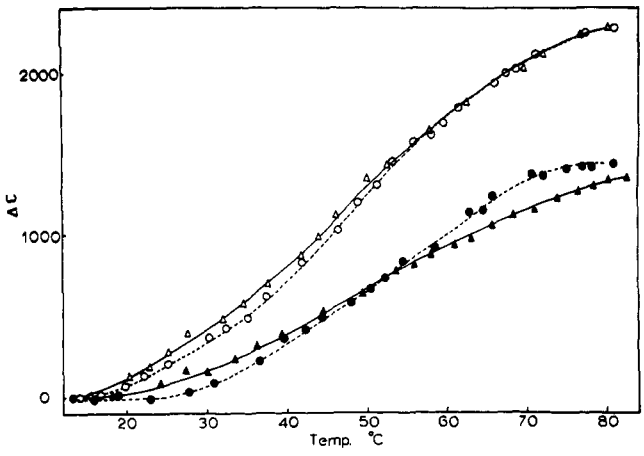

FIGURE 1: Absorbance–temperature profile of R17 RNA and globin mRNA in 0.01 M Tris-HCl–0.01 M NaCl–0.001 M EDTA (pH 7.5): R17 RNA at 260 (△) and 280 nm (▲); globin mRNA at 260 (○) and 280 nm (●). The results are expressed as the increment in the molar extinction coefficient at the two wavelengths.

Results

Variation of optical density as a function of temperature. The melting curves of R17 RNA and rabbit globin mRNA in 0.01 M Tris–0.01 M NaCl–0.001 M EDTA (pH 7.5) measured at 260 and 280 nm are shown in Figure 1. The assumptions discussed by Gould and Simpkins (1969) were used to analyze the increments in absorbance in order to get values for the content of base pairs and the compositions of the paired and unpaired regions of the RNAs. The values are listed in Table I. R17 RNA as well as globin mRNA have in this buffer and at 28° approximately the same helix content of about 65–70%. However, there is a significant difference in the amount of nonbase paired A and U residues between both mRNAs. More A residues are nonbase paired in globin mRNA than in R17 RNA, whereas the opposite observation is made for the U residues. In addition, the percentage of G·C base pairing is slightly smaller for R17 RNA than for globin mRNA. With respect to the transition profiles they are on a first approximation of sigmoidal character, although one can on close inspection distinguish a number of inflections, like in the case of 30S and 19S ribosomal RNA from rabbit reticulocytes (Gould and Simpkins, 1969).

Circular dichroism of R17 RNA and globin mRNA. CD spectra of R17 RNA and globin mRNA were recorded in two different buffer systems for comparative purposes and the results are shown in Figures 2a,b and 3. In Figure 2a both RNAs were measured in 0.01 M Tris–0.01 M NaCl–0.001 M EDTA (pH 7.5), the same buffer as that used previously for the optical density melting curves. Although the line shapes of both RNAs are similar and are of the nonconservative type, subtle differences exist between both CD curves with respect to the positions of the crossover points, maxima, troughs, and the intensity of the bands. The main positive circular band at about 267 nm is significantly higher for globin mRNA than for R17 RNA. It is also interesting to note that the low intensity negative band at about 298 nm is absent in both RNAs (*cf.* Figure 3). The dashed curve in Figure 2a is a calculated CD spectrum, which was obtained through procedures described in the method part. Incidentally, the globin mRNA CD spectrum can also be experimentally mimicked in the above buffer solution containing 92% of R17 RNA and 8% of poly(A) (*cf.* Figure 2b).

In a buffer system of higher ionic strength, containing 4×10^{-3} M Mg^{2+}, the similarity of the CD spectra for both RNAs is close (Figure 3). The intensity of the main positive band is virtually the same in both cases. The peak position of the R17 RNA is shifted slightly to the blue relative to that of globin mRNA. R17 RNA as well as globin mRNA exhibit now a low intensity negative band at 298 nm. Comparing these results with those in the buffer system containing no Mg^{2+} reveals that both RNAs have their main positive band shifted toward the blue by 1–2 nm in the presence of Mg^{2+}. However, the intensity of this band changes only in the case of R17 RNA, whereas Mg^{2+} seems to have no effect on the intensity of the globin mRNA main positive band.

Variation of Circular Dichroism as a Function of Temperature. In an attempt to compare the stability of R17 RNA and globin mRNA with and without the addition of Mg^{2+}, the temperature dependence of the main positive circular dichroism band was monitored. In Figure 4 the CD melting curves of both RNAs are shown in 0.01 M Tris–0.01 M NaCl–0.001 M EDTA (pH 7.5). Sigmoidal heating curves for $[\theta]$ at 266 nm *vs.* temperature are observed with a midpoint (T_m) at about 46° for R17 RNA as well as for globin mRNA. It can also be noticed that the residual ellipticity is higher for globin mRNA than for R17 RNA. With respect to the red shift of the peak

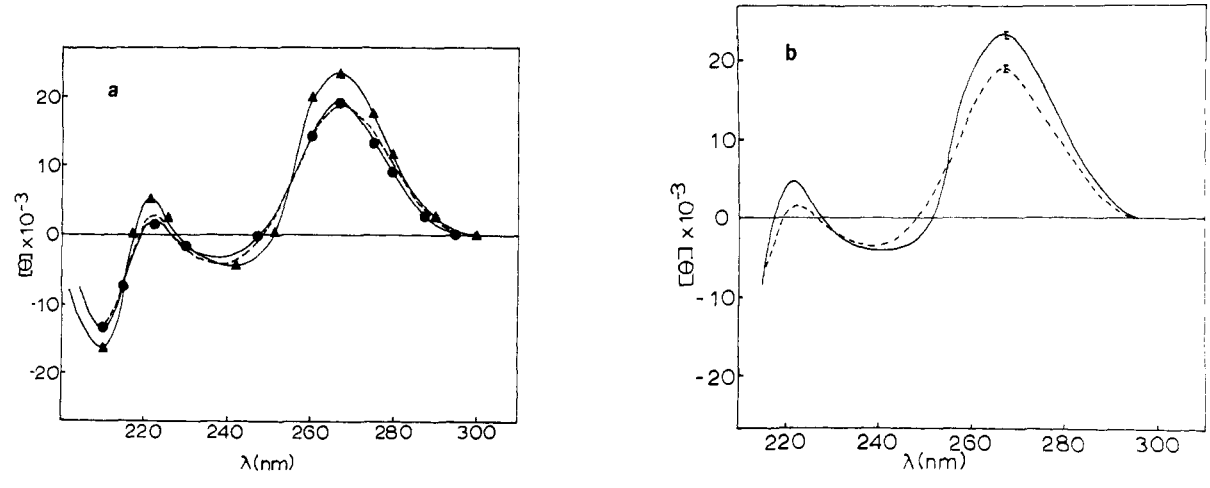

FIGURE 2: (a) CD spectra of R17 RNA (●) and globin mRNA (▲) in 0.01 M Tris-HCl–0.01 M NaCl–0.001 M EDTA (pH 7.5) at 28°. The measurements were performed in triplicate. The error at 265 nm is ±2%. (- - -) is a calculated CD spectrum obtained through procedures described in the method part. (b) CD spectra of R17 RNA (- - -) and a mixture (—) consisting of 0.92 and 0.08 mole fraction of R17 RNA and poly(A), respectively, in same buffer as Figure 2a.

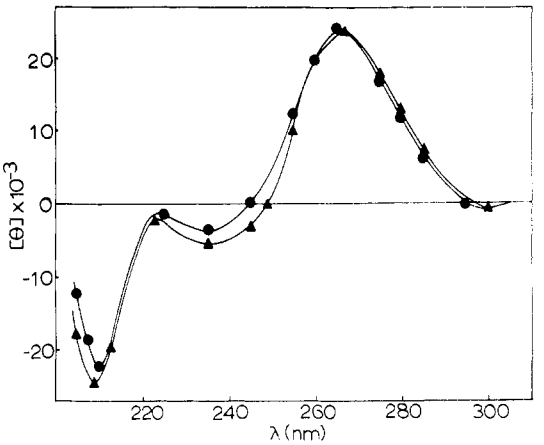

FIGURE 3: CD spectra of R17 RNA (●) and globin mRNA (▲) in 0.01 M Tris-HCl–0.04 M NaCl–4 × 10^{-3} M Mg^{2+} (pH 7.5) at 28°. The measurements were performed in triplicate. The error at 265 nm is ±2%.

of the intense positive band there is a small difference in particular at the high-temperature end. The red shift seems to be terminated at about 272 nm for globin mRNA, whereas for R17 RNA the peak is close to 274 nm. The CD melting curves of R17 RNA and globin mRNA in the presence of 4 × 10^{-3} M Mg^{2+} are shown in Figure 5. Considering the variation of [θ] at 266 nm (left ordinate) two distinct melting regions are apparent for both RNAs. In the case of globin mRNA one region lies below 42° having less of a slope than the one above 42°. For R17 RNA the breaking point is about 48°. Before the sharp breaking point the transitions occur with little change in [θ]. It can also be seen that in the melting region with the larger slopes R17 RNA melts at a higher temperature than globin mRNA. On the right ordinate of Figure 5 the shift of the peak ellipticity is plotted for both melting curves. In that case the difference between the melting behavior of both RNAs is even more pronounced. The shift

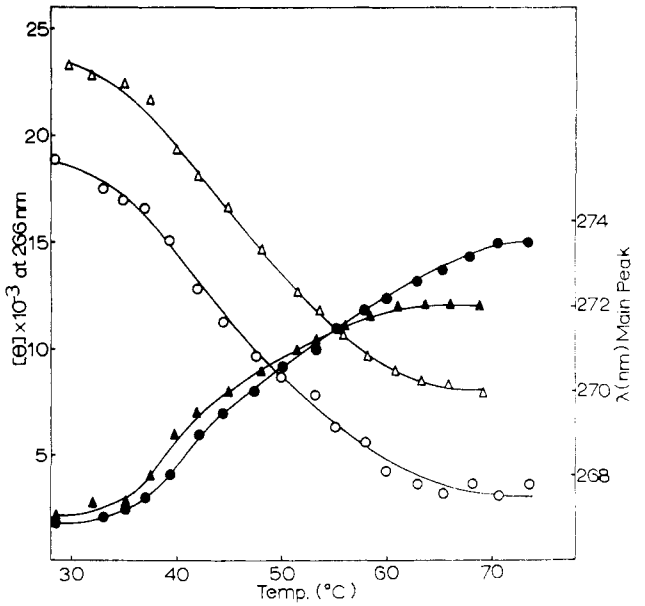

FIGURE 4: CD melting curves of R17 RNA and globin mRNA in 0.01 M Tris-HCl–0.01 M NaCl–0.001 M EDTA (pH 7.5). The melting studies were done in duplicate. Two parameters are plotted: on the left ordinate the peak residue ellipticity at 266 nm for R17 RNA (○), and globin mRNA (△); on the right ordinate the red shift of the peak of the intense positive band for R17 RNA (●) and globin mRNA (▲).

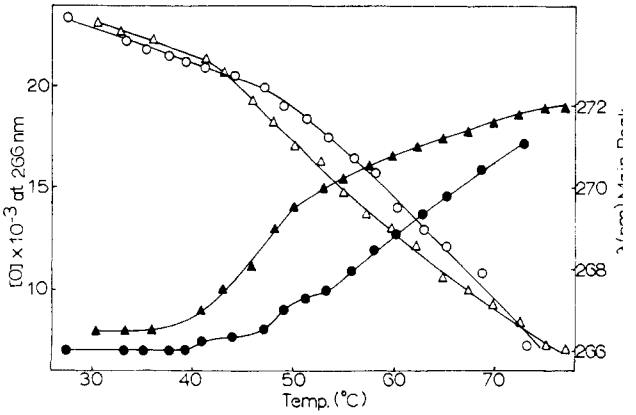

FIGURE 5: CD melting curves of R17 RNA and globin mRNA i 0.01 M Tris-HCl–0.04 M NaCl–4 × 10^{-3} M Mg^{2+} (pH 7.5). Th melting studies were done in duplicate. Two parameters are plotted on the left ordinate the peak residue ellipticity at 266 nm for R1 RNA (○) and globin mRNA (△); on the right ordinate the re shift of the peak of the intense positive band for R17 RNA (●) an globin mRNA (▲).

in wavelength of the band maximum consists of two distin guishable regions for globin mRNA. Between 40 and 50° large change in the peak position is observed, whereas abov 50° the slope of the curve is considerably smaller. Study of th peak shift curve of R17 RNA reveals two small cooperativ shifts with transition midpoints of about 40 and 49°, followe by a larger one with a transition midpoint of greater than 65° A similar observation has already been reported for R1 RNA in the presence of Mg^{2+} in a different buffer system The corresponding transition values were 37 and 46° in 0. × SSC buffer (0.015 M NaCl–0.0015 M sodium citrate (pH 7.0)) (Phillips and Bobst, 1972). The original CD spectra ca be restored for both RNAs. Even the 298-nm band can b obtained back if the RNAs are allowed to renature at 8–10 for about 12 hr.

Discussion

The analysis of the temperature–absorbance profiles i terms of compositions of double helices indicates (Table I that without the addition of Mg^{2+} the percentage of bases i base-paired regions is similar for both mRNAs. In terms o thermal stability both seem to have similar overall meltin properties. Both melting curves show discontinuities whic slightly differ from one another. It is believed that the dis continuities represent separate populations of double helices As to be expected, the secondary structure is to a certain exten different for both RNAs. The major difference, however, i found in the considerable large amount of nonbase paire adenylic acid residues for globin mRNA. About 7% mor adenylic acids are free at 28° in the mammalian messenge than in the bacteriophage RNA. It is tempting to assume tha this is the amount present as poly(A) in globin mRNA. Usin polyacrylamide gels Gaskill and Kabat (1971) determined molecular weight of 220,000 for rabbit mRNA. Thus, th free poly(A) stretch should be about 40–50 nucleotides lon Incidentally, this estimation would coincide with the valu found for the adenylate rich sequence in mouse globin mRNA In that case the adenylate sequence was determined to be abou 50 nucleotides long based upon its migration relative t poly(A) standards (Morrison *et al.*, 1973).

It is well established that in an RNA chain the optica activity of single-stranded structures might be large, whereas the hypochromicity is small (Boedtker, 1967; Gratzer, 1966

For the above analysis of the temperature–absorbance profiles the contributions from single-stranded melting were neglected. For that reason it seemed worthwhile to analyze both RNAs also by CD spectroscopy. The CD band of about 265 nm was carefully monitored, since its relation to the secondary structure of RNA had been the objective of several studies. It was suggested that a large red shift of this band, accompanied by a great decrease in ellipticity, indicates a disruption of nearly all base stacking as well as base-pairing interactions (Cantor *et al.*, 1966; Vournakis and Scheraga, 1966; Yang and Samejima, 1969). If the red shift of the band is coupled with only a small decrease in its magnitude, it is assumed that this is due mainly to the breaking of hydrogen bonds between base pairs.

In the buffer system containing no Mg^{2+} the intensity of the main positive band is unusually high for globin mRNA as compared to that of R17 RNA (Figure 2a). This could indicate that in the first case a greater proportion of residues are stacked without requiring additional hydrogen bonding to hold the bases in a helical conformation. There is agreement that at neutral pH poly(A) and its oligomers exist as single strand helices with partially stacked bases (Brahms *et al.*, 1966). In the presence of Mg^{2+} the CD spectra of both RNAs is very similar in terms of band intensities and peak position, suggesting that the degree of stacking remains approximately the same for globin mRNA in both buffer systems. In both cases the main positive peak undergoes a small blue shift indicating the formation of some more base pairs. At this point one expects the RNAs to have some well-defined tertiary structure besides the secondary structure already found in the buffer system without Mg^{2+}.

By using the CD data analysis described in the method section, it was attempted to calculate the average size of the poly(A) stretch in globin mRNA. The best fit shown in Figure 2a was obtained with $F = 0.08$. This means that an average of 8% of the nucleic acid residues form a homopolymer of adenylic acid building blocks in globin mRNA. If the molecular weight mentioned above is used for globin mRNA, the average size of poly(A) stretches would consist of 40–60 residues. Although the calculations are based on many approximations, it is interesting to note that the average size of the poly(A) is in the same neighborhood as the one estimated from optical density data.

With respect to the CD melting curves (Figures 4 and 5) it is obvious that the presence of Mg^{2+} does not only increase the stability of the helices, but in addition several cooperatively melting regions can now be distinguished. It can also be noticed that the melting pattern is less complex for globin mRNA than for the polycistronic R17 RNA. The CD changes in the low-temperature range might result from the melting of the tertiary structure, although melting of less stable hairpins would lead to similar CD observations. The CD melting profile is considerably simplified in the absence of Mg^{2+} and is similar for both RNAs. It is believed that in this buffer system the denaturation consists essentially of secondary structure to random coil transitions.

In summary, the present investigation shows that in an appropriate buffer system one can distinguish by spectroscopic means mRNAs containing poly(A) stretches from those without such sequences. Additionally, the data suggest that it is possible to get an estimate of the average size of the poly(A) in the poly(A) containing mRNAs.

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