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Determination of the Extent of Secondary Structure in Chick Embryo Procollagen Messenger RNA†

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ABSTRACT: The secondary structure of highly purified chick embryo procollagen mRNA has been investigated by thermal denaturation and by reaction with formaldehyde. Thermal denaturation parameters of procollagen mRNA have been compared to ribosomal RNAs and to a synthetic random copolymer. It is concluded that procollagen mRNA contains considerable secondary structure. Stacking interactions, calculated from the melting of procollagen mRNA previously reacted with 1% formaldehyde, account for about 10% of secondary structure. Kinetics of reaction with formaldehyde and thermal denaturation of procollagen mRNA in 4 M guanidinium chloride, a solvent which markedly reduces stacking interactions without affecting significantly hydrogen bond formation, show that about 49% of bases are present in a double helical configuration. Contrary to what is observed in the random sequence copolymer, the rise in temperature determines melting of hairpin loops progressively richer in G·C base pairs. The number of base pairs/hairpin loop has been estimated as 6. Assuming 4500 as the minimal number of nucleotides/mRNA and neglecting the contribution of poly(A) to hairpin loop formation, a minimum of 130 such hairpin loops are present in procollagen mRNA. $\Delta A_{260}/\Delta A_{280}$ for procollagen mRNA is in good agreement with the value predicted by a linear relationship between this ratio and base composition of any RNA species.

Ribosomal RNAs, as well as transfer RNAs, present extensive hydrogen bonding and base stacking interactions, leading to a high degree of secondary structure (Fresco et al., 1963). A comparable amount of structural complexity has also been shown for viral RNAs (Min Jou et al., 1972; Billeter et al., 1969). However, since random RNA sequences can contain as much as 60% hydrogen bonding (Fresco et al., 1960; Gralla and DeLisi, 1974) it is necessary, when studying the conformation of mRNA, to exclude chance base pairing as a cause for the observed secondary structure.

Studies have indicated that in R_{17} mRNA the initiator codon AUG is enclosed in a hairpin loop (Steitz, 1969). Other studies have shown that faithful translation of polycistronic mRNA can be altered by heat denaturing the message (Lodish and Robertson, 1969). It has also been suggested that the conformation of mRNA might play a role in its stability in the cell (Klambt, 1975).

Careful analysis of the structure of hemoglobin mRNA (Holder and Lingrel, 1975) has indicated the existence of a highly ordered structure, determined by about 60% hydrogen bonds. Three distinct helical domains with respect to base composition were evident. Analysis of the structure of ovalbumin mRNA (Van et al., 1976) has also shown the presence of short hairpin loops differing from the whole molecule in

† From the Laboratório de Oncologia Experimental, Faculdade de Medicina, Universidadé de São Paulo, São Paulo, Brazil. *Received February 3, 1977.* This work was aided by grants from Funsdação de Amparo à Pesquisa do Estado de São Paulo BioQ, Conselho Nacional de Pesquisas (CNPq), and Hospital das Clínicas da Faculdade de Medicina, da Universidade de São Paulo.

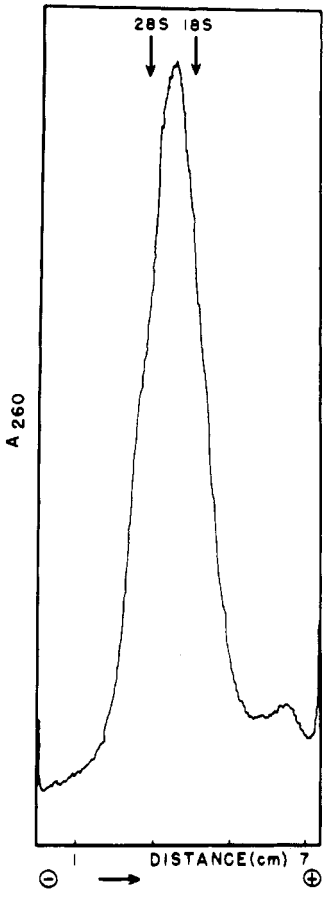

FIGURE 1: Agarose gel electrophoretic profile of procollagen mRNA.

regard to their base composition. Thus, in these two instances chance base pairing seems to be excluded by the experimental evidence presented.

Several workers have reported procollagen mRNA activity (Benveniste et al., 1973, 1976; Boedtker et al., 1974; Harwood et al., 1974) and even partial mRNA purification (Boedtker et al., 1976) in a number of RNA preparations obtained from embryonic chick tissues.

Previous work from our laboratory has shown that collagen synthesizing polysomes from chick embryos could be selectively isolated, because of their size, by low-speed centrifugation (Wang et al., 1975a). Procollagen mRNA obtained from these structures has been characterized by in vitro translation in a heterologous cell-free system (Wang et al., 1975b) and by determination of its size and base composition (Salles et al., 1976). We have now examined the secondary structure of procollagen mRNA, under conditions in which the contribution of stacking interactions and base pairing can be separately evaluated.

Materials and Methods

Isolation of collagen synthesizing polysomes, RNA extraction, mRNA purification, and agarose gel electrophoretic analysis, as well as criteria for determining the purity of procollagen mRNA, have already been reported (Wang et al., 1975a,b; Salles et al., 1976). Briefly, collagen-synthesizing polysomes, characterized as such by the behavior of molecular sieve and ion-exchange column chromatography of its in vitro translation product, as well as by its proline content and susceptibility to collagenase digestion, were isolated by low-speed centrifugation (40 000g × 60 min) from the post-mitochondrial supernatant of chick embryo homogenates (Wang et al., 1975a). Procollagen mRNA extracted from these particles and purified by oligo(dT)-cellulose chromatography was characterized by translation in a heterologous cell-free system. The translation product co-chromatographed with carrier collagen both in molecular sieve and ion-exchange chromatography, was very deficient in tryptophan, and was almost completely (92%) susceptible to collagenase digestion, under conditions which precluded nonspecific protease activity (Wang et al., 1975b). Finally, procollagen mRNA purified by a large scale method used also in the present work and by an analytical method which completely precludes rRNA contamination displayed the same electrophoretic mobility and base composition (Salles et al., 1976).

Ultraviolet Absorbance-Temperature Profiles. RNA samples were dissolved in 10 mM sodium phosphate buffer (pH 7.4), containing 0.1 M NaCl unless otherwise specified. Absorbance measurements were made with a Zeiss DMR 21 recording spectrophotometer equipped with a Colora thermostated water bath for temperature control.

The temperature inside the sample cell was calibrated using a thermocouple inside the reference cuvette. All temperature studies were corrected for solvent thermal expansion. Absorbance-temperature profiles were obtained by raising the temperature about 5 °C at a time, waiting 15 min before measuring the absorbance. Because the experiments took 3 h, the cells containing both solutions and solvent were weighed before and after each experiment to check for loss due to evaporation. Using stoppered quartz cells, less than 0.3% loss was observed in any experiment. The value for the molecular (ϵp) residue extinction coefficient was obtained by alkaline hydrolysis (Cox et al., 1970).

Kinetics of Reaction with Formaldehyde. Formaldehyde was added to a final concentration of 1% to a solution of procollagen mRNA in 10 mM phosphate buffer (pH 7.4), containing 0.1 M NaCl, and reaction kinetics were followed at 270 nm in an Acta III recording spectrophotometer. Room temperature was kept at 25 ± 1 °C. A_{270} after 24 h of reaction was taken as E_∞.

Results

Figure 1 shows the electrophoretic profile of the procollagen mRNA fraction used in the present experiments.

The UV absorption spectrum of a polynucleotide depends on conformation because of the interaction between neighboring base residues. Thus, it can yield information about the structure of these molecules.

In the present study three parameters of the denaturation spectrum were examined: (a) the "melting range" (i.e., the temperature range in which changes in structure take place; (b) the breadth of the melting range, gauged from the interquartile range (the temperature range over which A changes from 0.25 to 0.75 of the total increment observed on heating from 25 to 95 °C); (c) the ratio $\Delta A_{260}/\Delta A_{280}$, that is, the ratio between increments in absorbance at 260 and 280 nm respectively, when the sample is brought from 25 to 95 °C.

Figure 2 shows changes in the absorption spectrum of procollagen mRNA heated to 95 °C. Assuming that a 100% double-stranded structure presents a hypochromicity (the reduced absorbance of the native form relative to that of its denatured form) of 0.3 (Boedtker, 1967), the calculated hypochromicity of our mRNA fraction, 0.175, corresponds to 58% structure. It is also evident from Figure 2 that the T_m of the melting transition is dependent upon salt concentration, suggesting that changes in double helical secondary structure make a large contribution to A.

The nucleotide composition of helical segments melting over

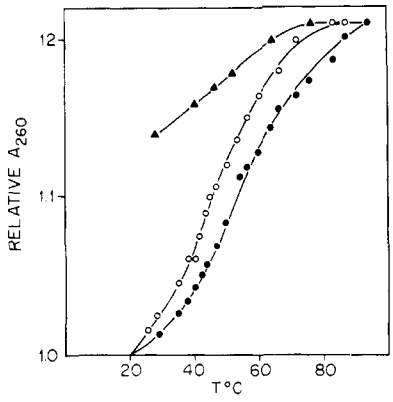

FIGURE 2: Thermal denaturation profile of procollagen mRNA: (●) procollagen mRNA in 0.1 M sodium phosphate buffer (pH 7.4); (○) procollagen mRNA in 0.01 M sodium phosphate buffer (pH 7.4); (▲) procollagen mRNA in 0.1 sodium phosphate buffer (pH 7.4) after reaction with 1% formaldehyde for 15 min at 85 °C.

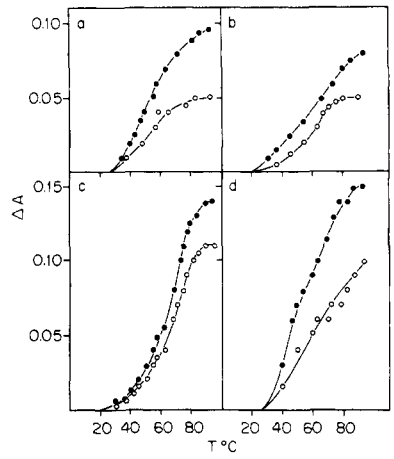

FIGURE 3: Thermal denaturation profile of natural RNAs. (a) Procollagen mRNA in sodium phosphate buffer; (b) procollagen mRNA in 4 M guanidinium chloride–0.01 M sodium phosphate buffer (pH 7.4); (c) 28S rRNA; (d) 18S rRNA: (●) A_{260}; (○) A_{280}. In all experiments A_{260} at room temperature = 0.75.

a particular temperature range can be estimated assuming that only G·C and A·U pairs are present in these segments and that each base pair contributes independently to the denaturation spectrum, irrespective of its nearest neighbors (Fresco et al., 1963). Thus, the fraction of guanine and cytosine residues present in double helical segments is measured by the ratio $\Delta A_{260}/\Delta A_{280}$ (Felsenfeld and Sandeen, 1962; Fresco et al., 1963; Cox, 1966), according to eq 1 (Cox, 1966).

$$f_{AU}/f_{FC} = 0.81(\Delta A_{260}/\Delta A_{280}) - 0.46 \quad (1)$$

If the nucleotide sequence is near random, a constant value of the $\Delta A_{260}/\Delta A_{280}$ ratio should be found over the entire melting range. Any deviation from this expected behavior must be attributed to the presence of clusters of particular nucleotides (Cox et al., 1976).

Figure 3 and Table I summarize the results of these determinations. As the temperature is increased a change in the ratio occurs, going from 2.10 for the first quartile to 1.78 for the last quartile; for 28S rRNA (Figure 3c) and 18S rRNA (Figure 3d) corresponding values were 1.26 and 1.80 for the first quartile and 1.06 and 1.18 for the last quartile, respectively.

A linear relationship exists between the T_m (defined as the midpoint of the transition from a bihelical to an amorphous form) and N, the number of base pairs/helical segment, when $N \leq 20$ (Kallenbach, 1968). Thus, N can be calculated by eq 2 (Cox, 1970a):

$$N = \frac{1.5 \ln b + \ln \tau - 1.5 \ln 2}{(1/T_{m(A\cdot U)} - 1/T_m)\Delta H/R + f_{(G\cdot C)} \ln K} \quad (2)$$

where b is the number of unpaired residues/hairpin loop, τ is the equilibrium constant for stacking two adjacent base pairs upon each other, ΔH is the ethalpy/mole of nucleotide pair for increasing the length of the helix by one base pair, and $\ln K = \Delta H/R(1/T_{m(G\cdot C)} - 1/T_{m(A\cdot U)})$. Since $\ln K$, Δ, τ, and $T_{m(A\cdot U)}$ are known (Kallenbach, 1968) as well as b (= 5; Rubin and Kallenbach, 1975) and T_m and $f_{(G\cdot C)}$ are experimentally determined, N is found to be equal to 6. In all the above calculations the contributions of stacked but unpaired residues have been neglected. Such a contribution can be evaluated in two ways: (a) first, the hypochromism of base residues in single-stranded regions is preferentially diminished by 4 M guanidinium chloride, which does not affect the hypochromism of double helical structures (Cox and Kanagalingam, 1968). Secondly, hypochromism of stacked residues is studied utilizing mRNA which has been pretreated with formaldehyde to prevent the formation of base pairs.

TABLE I: Some Parameters of the Melting Profile of Procollagen mRNA and Other Polynucleotides.[a]

	Melting range (°C)	Interquartile range (°C)	T_m (°C)	$\Delta A_{260}/\Delta A_{280}$
Procollagen mRNA	25–93	22	54	1.94
	25–37			2.10
	59–93			1.78
28S rRNA	25–93	20	67	1.17
	25–54			1.26
	74–93			1.06
18S rRNA	25–93	24	55	1.49
	25–44			1.80
	68–93			1.18
Poly(AGUC)	25–90	29	48	1.73
	25–43			1.75
	72–90			1.71

[a] The solvent was 0.1 M sodium phosphate buffer (pH 7.4) for all polynucleotides.

Figure 3b shows the melting profile of procollagen mRNA in the presence of 4 M guanidinium chloride. A sharper transition profile is obtained, compared to that shown in Figure 3a. Also, whereas the increase in absorbance at 280 nm is similar to that shown in Figure 3a, that of the absorbance at 260 nm was considerably reduced, leading to a calculated hypochromicity of 0.145 equivalent to 49% structure.

Figure 2 shows the absorbance–temperature profile of procollagen mRNA after reaction with formaldehyde. It is clear that the formaldehyde derivative is much less hypochromic than unreacted procollagen mRNA. Furthermore, the linear dependence on temperature is characteristic of the melting of single-stranded stacked base residues (Boedtker, 1967).

An independent assessment of the extent of hydrogen bonding can be obtained through kinetics of reaction with 1% formaldehyde (Stevens and Rosenfeld, 1966). The reaction can be followed at 270 nm in 0.1 M sodium phosphate buffer (pH 7.4) at 25 °C. The reaction is pseudo first order with respect to formation of methylol adducts (Penniston and Doty, 1963). Figure 4 shows a plot of the extent of reaction of formaldehyde with procollagen mRNA and an equivalent mixture of ribonucleotides.

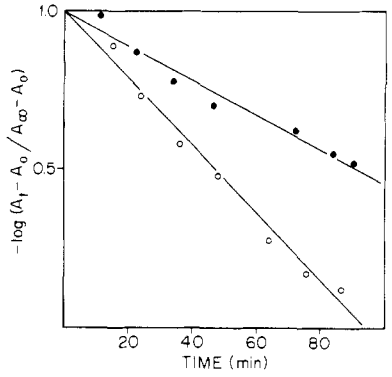

FIGURE 4: Kinetics of reaction with formaldehyde. Procollagen mRNA (●) or an equivalent mononucleotide mixture (○) reacted with formaldehyde as outlined under Materials and Methods. Reaction kinetics were followed at 270 nm.

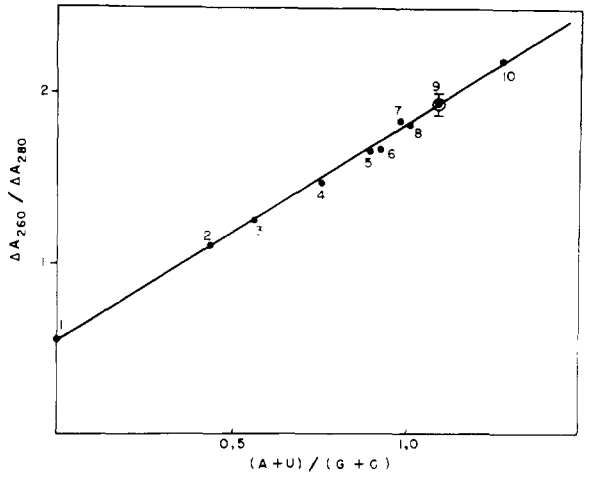

FIGURE 5: Dependence of $\Delta A_{260}/\Delta A_{280}$ on nucleotide composition (A + U)/(G + C). $\Delta A_{260}/\Delta A_{280}$ was calculated from the denaturation spectrum obtained by heating polyribonucleotides to 93 °C: (1) poly(G·C) (Fresco et al., 1963); (2) 28S rRNA; (e) *Escherichia coli* 5S RNA (Boedtker and Kelling, 1967); (4) 18S rRNA; (5) double helical RNA from *Penicillium chrysogenum* (Cox, 1970); (6) ovalbumin mRNA (Van et al., 1976); (7) poly(AGUC); (8) hemoglobin mRNA (M. Brentani, unpublished results); (9) procollagen mRNA; (10) TMV RNA (Fresco et al., 1963).

The pseudo-first-order rate constants derived from the slopes are 2.02×10^{-2} min^{-1} for free mononucleotides and 0.99×10^{-2} min^{-1} for procollagen mRNA. Assuming that formaldehyde reacts at the same rate and to the same extent with available bases in procollagen mRNA as it does with free bases, a comparison of the rate constants indicates that about 49% of the bases are not available for reaction under the conditions used, in close agreement with the value calculated above from the data shown in Figure 2.

Taking into consideration the contribution of single-stranded stacking, the fraction of residues located in double-stranded segments of mRNA can also be estimated by eq 3 (Cox, 1970a):

$$f_{dh} = (\epsilon_s - \epsilon)/(\epsilon_s - \epsilon_{dh}) \quad (3)$$

where ϵ_s is the extinction found at room temperature after heating at 90 °C in the presence of 1% formaldehyde (Cox, 1970a) (ϵ_s = 9000); ϵ is the extinction of native procollagen mRNA and equal to 7800 and ϵ_{dh} is the extinction of the appropriate bihelical analogue (Cox et al., 1976), calculated according to the formula (Cox, 1970a):

$$\epsilon_{dh} = \epsilon_{am} - |\Delta\epsilon_{(G\cdot C)} f_{(G\cdot C)} + \Delta\epsilon_{(A\cdot U)} f_{(A\cdot U)}|$$

TABLE II: Base Composition of Helical and Nonhelical Regions of Procollagen mRNA.

Base	Base paired (%)	Non-base-paired (%)	Total (%)
A	15.3	16.7	32
U	15.3	4.7	20
G	14	11	25
C	14	9	23
A·U	30.6	21.4	52
G·C	28	20	48
A·U·G·C	58.6	41.4	100

where ϵ_{am}, the extinction of the amorphous form, determined at 90 °C was 9500. $\Delta\epsilon_{(A\cdot U)}$ and $\Delta\epsilon_{(G\cdot C)}$ were taken to be 3900 units (Fresco et al., 1963) and 1800 units (Cox, 1966), respectively. ϵ_{dh} was equal to 6600.

Calculated f_{dh} was 49%. Assuming that there are 17 nucleotides/hairpin (N = 6; b = 5) and a molecular weight of 1.6×10^6, the amount of double helical structure corresponds to 132 hairpin loops.

Based on the assumption that the nucleotide composition of double helical regions reflects the overall nucleotide composition of any RNA species, a linear relationship has been demonstrated between $\Delta A_{260}/\Delta A_{280}$ and A + U/G + C given by eq 4 (Cox, 1970b).

$$\Delta A_{260}/\Delta A_{280} = 1.38(A + U/G + C) + 0.45 \quad (4)$$

Figure 5 shows the results of such an analysis and it is clear that the experimentally observed value for $\Delta A_{260}/\Delta A_{280}$, 1.94 ± 0.2, is in close agreement with 1.95, the value predicted by the known base composition of procollagen mRNA (Salles et al., 1976). The fact that data for double helical RNAs as well as for single-stranded species were given by the same equation, i.e. fell on the same straight line, validates the assumption made.

Since the extent of double helical structure can be estimated, thermal melting profiles can be analyzed using the assumptions set out by Gould and Simpkins (1969) to yield further information on the content of base pairs and the base composition of paired and unpaired regions of the molecule (Table II). It is clear that there is a large excess in adenine residues (16.6%) as compared to uracyl (4.6%) in the unpaired regions, whereas guanine and cytosine residues are evenly distributed in helical and nonhelical regions.

Discussion

The effect of salt concentration on T_m, depicted in Figure 2, shows that hydrogen bonds are very important to the maintenance of secondary structure. At high ionic strength the repulsion between negatively charged phosphate residues located on opposite strands of the double helix is neutralized (Schildkraut and Lifson, 1965). As stressed above, it is very important to show that the high degree of secondary structure found (58%) is not due to chance base pairing. This is indicated by the variation in $\Delta A_{260}/\Delta A_{280}$ observed between the first and last quartiles (Figure 3 and Table I) and by the experimentally determined average length of the hairpin loops, slightly larger than those found in the random copolymer. The same conclusion is strengthened by the fact that the interquartile range for procollagen mRNA approximates those of the ribosomal RNA species and is smaller than that of the random copolymer (Table I).

The evaluation of stacking interactions has been performed in three different ways, with good agreement in the calculated values.

The reduction in the change in A_{260} observed on heating procollagen mRNA in 4 M guanidinium chloride–0.1 M sodium phosphate buffer (pH 7.4) agrees with the melting of double helical structures. Thus, the T_m was close to that found in 0.1 M sodium phosphate buffer and $\Delta A_{260}/\Delta A_{280}$ was found to depend on the temperature range and was slightly less than values calculated from the corresponding region of melting profiles obtained in phosphate buffer solutions (Cox, 1970a). The reduction in the change in A_{260} observed in the presence of this solvent has been attributed to the predominant participation of adenine residues in stacking interactions (Cox and Kanagalingam, 1967). The amount of secondary structure deduced in such a way agrees completely with that calculated from kinetics of reaction with formaldehyde (49%) (Figure 4). It is also worth noticing that the rate constants experimentally obtained, 2.02×10^{-2} min^{-1} and 0.99×10^{-2} min^{-1} for free nucleotides and for procollagen mRNA, respectively, are also in good agreement with those that can be calculated from the base composition of procollagen mRNA (Salles et al., 1976) and of its double helical regions (Table II) and the rate constants for hydroxymethylation of mononucleotides (Grossman et al., 1961).

The data from Figure 2 allow us to apply the equation for total hypochromicity:

$$h_T = (1 - x)h_{ss} + xh_{ds} \quad (5)$$

where h_{ss} and h_{ds} are the hypochromicities of completely single- and completely double-stranded RNA (Boedtker, 1967). h_T and h_{ss} were determined experimentally in the absence or presence of formaldehyde, respectively; h_{ds} was assumed to be 0.30. Thus, about 50% of bases are in double helical domains and 8% of the secondary structure is due to single-stranded stacked regions.

Comparison of thermal denaturation parameters of procollagen mRNA, 28S rRNA, and 18S rRNA (Figure 3 and Table I) shows that no significant contamination of the mRNA fraction occurs. Further evidence of the purity of our mRNA fraction is given in Figure 5 which shows that the experimentally determined $\Delta A_{260}/\Delta A_{280}$ for procollagen mRNA is in good agreement with that predicted from its base composition and the linear relationship given by eq 4 (see Results).

The poly(adenylic acid) sequence attached to the 3′ end of procollagen mRNA is 140 nucleotides long (Salles et al., 1976). Because of the size of the message (4500 nucleotides to code for 1500 amino acids presumed to be present in the primary translation product (Monson et al., 1975)) poly(A) can account for only 3% of the molecule, taking the unlikely assumption that there are no untranslated sequences in the mRNA molecule. Therefore, its contribution to the overall secondary structure can be neglected.

Acknowledgments

The authors are indebted to Mrs. Jacyra Martins and Mr. A. M. Olmo for their able technical assistance.

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