Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 338-346

CONTRIBUTION OF POLYADENYLIC ACID SEQUENCES TO THE MAINTENANCE OF SECONDARY STRUCTURE OF GLOBIN MESSENGER RNA

Mitzi Brentani, Cecília L.S. dos Santos, Francisco H.D'Abronzo and Ricardo R. Brentani

Fundação Centro de Pesquisa de Oncologia and
Laboratório de Oncologia Experimental
Faculdade de Medicina - Universidade de São Paulo
Caixa Postal 8100
São Paulo - Brasil

Received August 1,1979

SUMMARY

Purified rabbit hemoglobin mRNA (HbmRNA) has been characterized by cell-free translation in vitro and by denaturing (glyoxal) gel electrophoresis. Enzymatic deadenylation yields shorter molecules, susceptible of translation but unable to serve as a template for reverse transcriptase. Comparison between thermal denaturation parameters of intact and deadenylated HbmRNA in phosphate buffer alone before and after reaction with formaldehyde (90º x 10min) and in phosphate buffered 4M guanidinium chloride indicates a highly ordered secondary structure, 10% of which is given by single stranded base stacking, in intact HbmRNA. Deadenylated HbmRNA displays very little single stranded base stacking, suggesting that most of this contribution to the overall structure of intact HbmRNA is given by its poly(A) sequence.

INTRODUCTION

Poly(A) has been shown to be a natural cellular component (1) associated with mRNA (2). The biological significance of poly(A) remains a puzzle, despite a number of interesting models that have been put forward (3,4).

Information has begun to appear on the conformation of mRNA. The amount of secondary structure of hemoglobin mRNA (5), ovalbumin mRNA (6) and procollagen mRNA (7) has already been estimated. Data on the contribution of poly(A) to the molecular conformation of mRNAs is however still scanty. Comparison of the susceptibility of intact and deadenylated HbmRNA to nuclease S_1 digestion (8) has shown that it does not participate in double helix formation. This observation is in agreement with the absence of oligo(U) regions demonstrated by sequence analysis of HbmRNA (9).

Work on procollagen mRNA has indicated that very little single stranded base stacking occurs. This observation has been confirmed on HbmRNA (10) by optical and spectrophotometric methods. Since it is

0006-291X/79/170338-09$01.00/0
Copyright © 1979 by Academic Press, Inc.
All rights of reproduction in any form reserved.

known that poly(A) has a tendency to stack (11) we have tried to measure the amount of single stranded base stacking and to determine the contribution of poly(A) to the conformation of HbmRNA.

MATERIALS AND METHODS

RNase H (E.C. 3.1.4.34) and oligo(dT)12-18 were obtained from Miles Chem. Co., Elkhart, Indiana. AMV[1] reverse transcriptase was supplied by Dr. J.W. Beard (Life Sciences Inc. Gulfport, Florida). Cold nucleotide and deoxynucleotide triphosphates, Sephadex G-50, creatine phosphate (CP) and creatine phosphokinase (E.C.2.7.3.2) were obtained from SIGMA Chem.Co. St Louis,Missouri. Oligo(dT) cellulose (T3) was purchased from Collaborative Research. (^{35}S)-methionine (specific activity 627.7 Ci/mMol) and (^{3}H)-thymidine triphosphate (specific activity 21 Ci/mMol) were acquired from New England Nuclear. All other reagents were analytical grade and all solutions were made in twice-glass-distilled water and autoclaved before use, whenever possible.

Cellular fractionation and RNA purification. Rabbit globin mRNA was obtained by a cold phenol-chloroform extraction of reticulocyte polysomes and purified by two consecutive chromatographies on oligo(dT)cellulose columns (10). NaCl in application buffer was lowered to 0.2M as this reduces rRNA contamination(12).

Deadenylation of HbmRNA. The method of Sippl et al (13) was followed.

Polyacrylamide gel electrophoresis. Analyses were made in the presence of glyoxal as a denaturing agent (14).

In vitro aminoacid incorporation and mRNA reverse transcription. The exogeneous mRNA dependent wheat-germ cell-free system was used for in vitro protein synthesis (15). Following 60 min incubation at 30°C aliquots were either counted for radioactivity or analysed in SDS-polyacrylamide gels (16). Reverse transcription was carried out essentialy as described by Sippl et al (13).

Ultraviolet absorbance - temperature profiles and kinetics of reaction. Experimental conditions have already been described (7). The value for the molecular (εp) residue extinction coefficient was obtained by alkaline hydrolysis (17).

RESULTS AND DISCUSSION

Fig. 1 shows the electrophoretic profile of intact and deadenylated HbmRNA. By comparison with ribosomal RNA markers, the mobility of both molecules corresponds to molecular weights of 230,000 and 185,000 respectively. This difference can be attributed to the removal of 80 AMP residues from the 3' end. Both mRNA fractions display a single sharp band. A further measure of the purity of our HbmRNA preparation is given in Fig. 2, which shows that this material is able to direct the in vitro synthesis, by a wheat-germ cell-free system, of polypeptides which co-electrophorese with unlabeled carrier globin.

[1]Abbreviations: AMV: Avian Mieloblastosis Virus.

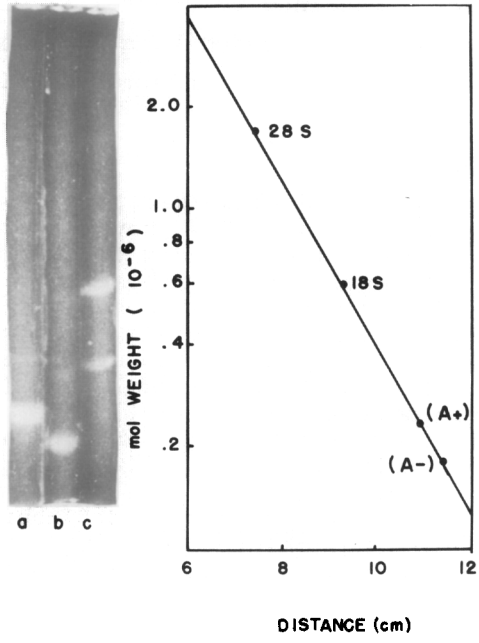


Figure 1. Polyacrylamide gel electrophoretical profile of HbmRNA. Intact and deadenylated HbmRNA, as well as reticulocyte rRNA were run in denaturing gels (18). Left panel - a) intact HbmRNA; b)deadenylated HbmRNA; c)rRNA. Right panel, plot of migration vs log molecular weight.

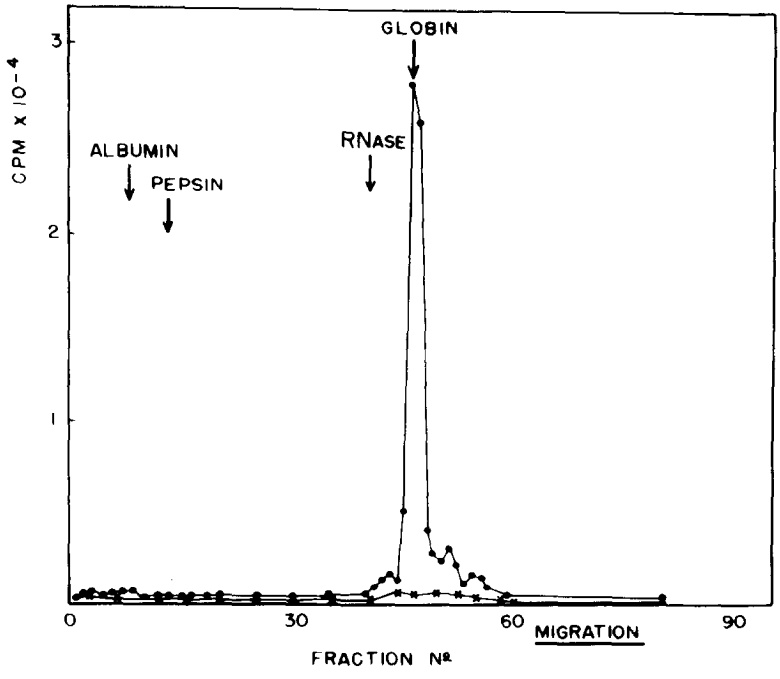


Figure 2. Gel electrophoretical profile of the product of in vitro translation of HbmRNA . The crosses refer to endogeneous incorporation by the wheat germ.

TABLE I

Translational and reverse transcriptional properties of HbmRNA

	Intact HbmRNA	Deadenylated HbmRNA
amino acid incorporation (c.p.m.)	31.491	36.304
reverse transcription (c.p.m.)	239.508	25.178

Table I shows the translational and reverse transcriptional capabilities of deadenylated HbmRNA as compared to those of intact mRNA. It is clear that there is no impairment of its ability to direct aminoacid incorporation by a wheat-germ cell-free system. On the other hand, digestion with nuclease H drastically reduces its ability to direct the in vitro synthesis of cDNA.

The fraction of G+C in bihelical regions melting to an unfolded form over a given temperature range has been estimated roughly by applying the equation (17):

$$f_{AU}/f_{GC} = 0.81\ (\Delta\varepsilon_{260}/\Delta\varepsilon_{280}) - 0,46 \qquad (1)$$

The $\Delta\varepsilon_{260}/\Delta\varepsilon_{280}$ ratio for HbmRNA (Fig. 3a) over the first quartile is 1.75, as opposed to 1.43 for the last quartile (Table II); the corresponding values for 18S rRNA (Table II) were 1.78 and 0.9 respectively. As one would expect, the $\Delta\varepsilon_{260}/\Delta\varepsilon_{280}$ ratio did not change apreciably over the entire melting range for polyAGUC (Table II). The results indicate the existence in HbmRNA to their base composition.

Besides the electrophoretical profiles of both our HbmRNA fractions and its translation products, another criterion of its purity can be given by estimation of its base composition calculated by the formula:

$$\Delta\varepsilon_{260}/\Delta\varepsilon_{280} = 1,38\ (A+U/G+C) + 0.45 \qquad (2)$$

which is applicable to double helical as well as single stranded RNAs (17). Taking values found in table II we find 52% GC, in close agreement with values reported in the literature (18).

The contribution of single-stranded base stacking can be evaluated in two ways: a) first, the hypochromism of base residues in single-stranded regions is preferentially diminished by 4M guanidinium chloride, which does not affect the hypochromism of

TABLE II

Some parameters of the melting profile of HbmRNA and other polynucleotides*

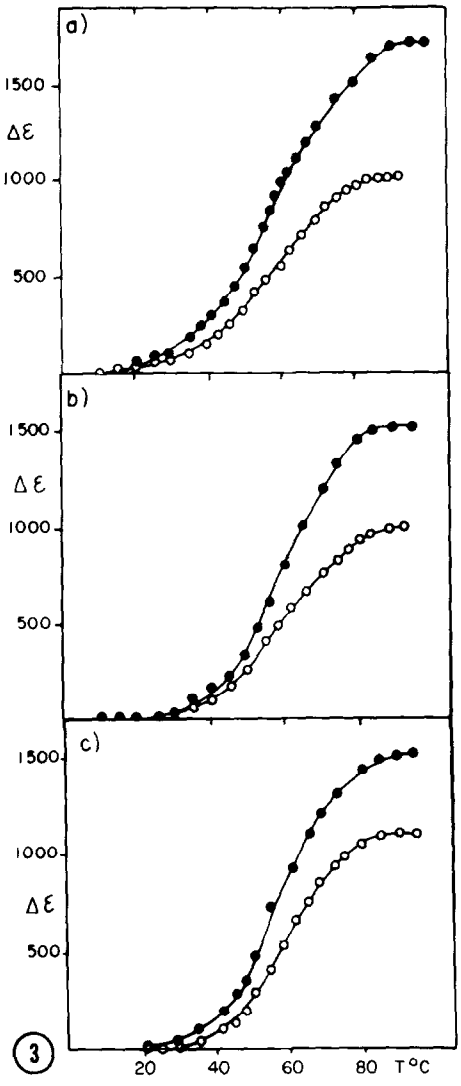
	Interquartile range	T_m (°C)	$\Delta\varepsilon 260/\Delta\varepsilon 280$	
HbmRNA	20 (42-62°C)	54	20-90°C	1.68
			20-42°C	1.75
			62-90°C	1.43
Intact mRNA in 4M guanidinium chloride	17 (50-67°C)	58	20-90°C	1.50
			20-50°C	1.58
			67-90°C	1.44
deadenylated HbmRNA	18 (47-65°C)	55	20-90°C	1.50
			20-47°C	1.70
			65-90°C	1.41
18S rRNA	16 (48-64°C)	57	20-90°C	1.50
			20-48°C	1.78
			64-90°C	0.90
Random poly(AGUC)	29 (43-72°C)	48	20-90°C	1.71
			20-43°C	1.75
			73-90°C	1.71

* the solvent was phosphate buffer 0.1M pH 6.8 for all polynucleotides, unless otherwise specified.

double helical structures (19). Secondly, hypochromism of stacked residues is studied utilizing mRNA which has been pretreated with formaldehyde to prevent the formation of base pairs.

Figure 3b shows the melting profile of HbmRNA in the presence of 4M guanidinium chloride. The T_m is close to that found in 0.1M sodium phosphate buffer but a sharper transition profile is obtained, compared to that shown in Figure 3a. Also, whereas the increase in absorbance at 280nm is similar to that shown in Figure 3a, that of the absorbance at 260nm is considerably reduced. This can be attributed to the predominant stacking of unpaired AMP residues (19). With the same reasoning applied to HbmRNA in phosphate buffer, the hypochromicity of HbmRNA in the presence of 4M guanidinium chloride corresponds to 52% structure, suggesting, because of the known effect of the solvent, that roughly 6% of the optically detectible structure of native HbmRNA is attributable to single stranded base stacking.

Fig. 3c shows that the melting profile of enzymatically deadenylated HbmRNA is strikingly similar to that of intact HbmRNA in 4M guanidinium chloride. There is consequently a close agreement between the respective denaturation parameters (Table II).



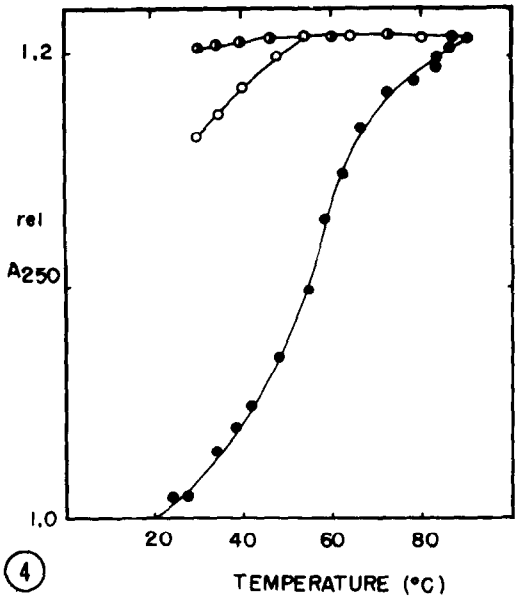


Figure 3. Melting profile of HbmRNA. (—●—), A_{260}; (—○—), A_{280}. a) Intact HbmRNA in 0.01M sodium phosphate buffer containing 0.1M NaCl. b) Intact HbmRNA in 0.01M sodium phosphate buffer containing 4M guanidinium chloride. c) Deadenylated HbmRNA in sodium phosphate buffer containing 0.1M NaCl. Deadenylation of HbmRNA was performed with the aid of ribonuclease H as described in the methods section.

Figure 4. Melting profile of intact HbmRNA before (—●—) and intact HbmRNA (—○—) and deadenylated HbmRNA (—●—) after reaction with 1% formaldehyde. Further experimental details are given in the methods section.

In fig. 4 we see the results of the absorbance studies carried out at 250nm on RNA before and after reaction with formaldehyde. Intact HbmRNA has a sigmoid melting curve before reaction; its formaldehyde derivative exhibits linear dependence on temperature,

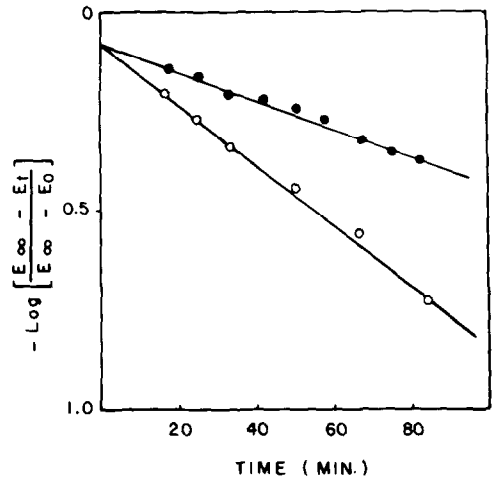


Figure 5. Kinetics of reaction of HbmRNA with formaldehyde. HbmRNA (—●—) or an equivalent mononucleotide mixture (—○—) reacted with formaldehyde as outlined in methods. Reaction kinetics were followed at 240nm.

characteristic of single stranded stacked bases (20). Deadenylated HbmRNA shows , on the other hand, very little hypochromicity, indicating that single stranded base stacking contributes very slightly to its secondary structure. After cooling to 20ºC, the original spectra were largely restored.

The data from fig. 4 allow us to apply the formula for total hypochromicity $h_T=(1-x)h_{ss} + h_{ds}$ where h_{ss} and h_{ds} are the hypochromicities of completely single and completely double stranded RNA. h_T and h_{ss} were determined experimentally in the absence and presence of formaldehyde respectively; h_{ds} was assumed to be 0,30. The calculated value for x (the fraction of nucleotides in double helical regions) is 0.53; thus correction for single stranded base stacking reduces the estimated helical content by 10%.

An independent assessment of the extent of hydrogen bonding can be obtained through kinetics of reaction with 1% formaldehyde (11). The reaction can be followed at 275nm in 0,1M sodium phosphate buffer (pH6.8) at 25ºC. The reaction is pseudo first order with respect to formation of methylol adducts (21). Fig. 5 shows a plot of the extent of reaction of formaldehyde with HbmRNA and an equivalent mixture of ribonucleotides.

The pseudo first order rate constants derived from the slopes are $2,36x10^{-2}min^{-1}$ for free mononucleotides and $1,26x10^{-2}min^{-1}$ for HbmRNA. Comparison of the rate constants indicates that about 53% of

the bases are not available for reaction under the condition used, in close agreement with the value calculated above from the data shown in Fig. 4. It is also worth noticing that the rate constants experimentally obtained, $2.02 \times 10^{-2} min^{-1}$ and 0.99×10^{-2} for free nucleotides and for HbmRNA respectively are also in good agreement with those that can be calculated from the base composition of HbmRNA (18) and the rate constants for hydroxymethylation of mononucleotides (22).

The amount of secondary structure deduced by melting HbmRNA in 4M guanidinium chloride agrees completely with that calculated from kinetics of reaction with formaldehyde (53%) (Fig. 3c). This results is consistent with the idea that the poly(A) segment is responsible for the major part of the base stacking seen in our UV hypochromism studies .

ACKNOWLEDGMENTS

This work was aided by grant 77/1521 from Fundação de Amparo à Pesquisa do Estado de São Paulo. The authors acknowledge the able technical assistance of Mr. A.M.Olmo and Miss S.M.Takeda. We are also deeply indebted to Prof. N.R.Kallenbach (University of Pennsylvania) for his carefull discussion of the manuscript.

REFERENCES

1. Brawerman, G., Gold, L. and Eisenstadt, J. (1963) Proc.Natl.Acad. Sci. US 50, 630-638.
2. Edmonds, M. and Caramela, M.G. (1969) J.Biol.Chem. 244, 1314-1324.
3. Sussman, M. (1970) Nature 225,1245-1246.
4. Darnell, J.E.,Philipson, L.,Wall,R. and Adesnik (1971) Science 174, 507-510.
5. Holder, J.W., and Lingrel, J.B. (1975) Biochemistry 14, 4209-4214.
6. Van, N.T., Holder, J.W., Woo, S.L.C., Means, A.R., and O'Malley, B.W. (1976) Biochemistry 15, 2054-2062.
7. Brentani, M., Salles, J.M.,and Brentani, R. (1977) Biochemistry 16, 5145-5149.
8. Vournakis, J.N., Flashner, M.S., Katopes, M.A., Kitos, G.A., Vamvakopoulos, N.C., Sell, M.S. and Wurst, R.M. (1976) Progr. Nucleic Acids Res. Mol. Biol.19, 233-252.
9. Proudfoot, N.J. and Brownlee (1976) Brit.Med.Bull.32, 251-256.
10. Brentani, M., Salles, J.M., Zinner, K., Faljoni, A. and Brentani, R. (1978) Biochem.Biophys.Res.Com. 83, 124-131.
11. Stevens, C.L. and Rosenfeld, A. (1966) Biochemistry 5, 2714-2721.

12. Hames and Perry, R.P. (1977) J.Mol.Biol. 109, 437-453.
13. Sippl, A.E., Stavrianopoulos, J.G., Schutz,G. and Feigelson,P. (1974) Proc. Natl. Acad.Sci. US 71, 4635-4639.
14. McMaster, G.K. and Carmichael, G.G. (1977) Proc.Natl.Acad.Sci. US 74, 4835-4838.
15. Roberts, B.E. and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. US 70, 2330-2334.
16. Stolf, A.M.S., D'Abronzo, F.H., Wang, L., Salles, J.M., Andrade, H.F., Silva, S.M.F. and Brentani, R. (1976) Bioch. Biophys. Res. Comm. 72, 1576-1584.
17. Cox, R.A., (1970) Biochem. J. 120, 539-547.
18. Williamson, R., Morrison, M., Lanyon, G., Eason, R. and Paul, J. (1971) Biochemistry 10, 3014-3021.
19. Cox, R.A. and Kanagalingam,K. (1968) Biochem. J. 108, 599-610.
20. Boedtker, H. (1967) Biochemistry 6, 2718-2727.
21. Penniston, J.T. and Doty, P. (1963) Biopolymers 1, 145.
22. Grossman, L., Levine, S.S. and Allison, W.S. (1961) J.Mol.Biol. 3, 47-60.