Interaction Between Polyuridylic Acid and Rabbit Globin Messenger Ribonucleic Acid

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Poly(U) binds to globin mRNA in 0.1 M-NaCl. Studies with ribonuclease digestion of this complex suggest that there are polyadenylate-rich sequences in the mRNA containing about 30–40 adenylate residues. The sequences appear to be homogenous and of approximately the same length for both α - and β -globin mRNA. They are most likely located at the 3' terminus of the molecule.

Polyadenylate sequences have been isolated by pancreatic ribonuclease digestion of rabbit globin mRNA (Lim & Canellakis, 1970). Chain lengths of 50–70 nucleotides were estimated from the mobility of these oligonucleotides relative to RNA on polyacrylamide gels. They represent about 10% of the mRNA molecule and contain 70% adenylic acid. More recently Burr & Lingrel (1971) and Hunt (1973) have identified polyadenylate sequences at the 3' termini of globin mRNA. The longest adenylate sequence so far determined is seven nucleotides (Hunt, 1973). In the present study globin mRNA has been hybridized with poly(U) to determine the size and distribution of polyadenylate sequences in mRNA. Pretreatment of mRNA with exonucleases was used to determine whether the poly(A) sequences are terminally located. The quantitative estimation of poly(U) bound is complicated by poly(A)·2 poly(U) triple-helix formation; an attempt has been made to allow for this by hybridization and nuclease treatment at high and low salt concentrations.

Experimental

Materials

G-³H-labelled poly(U) (sp. radioactivity 8.1 mCi/mmol and 7.0 mCi/mmol), 8-¹⁴C-labelled poly(A) (sp. radioactivity 2.15 mCi/mmol) and ribonuclease-free sucrose were obtained from Schwarz/Mann (Orangeburg, N.Y., U.S.A.). Poly(A) and spleen phosphodiesterase (EC 3.1.4.1) were from Sigma Chemical Co. (St. Louis, Mo., U.S.A.). Pancreatic ribonuclease (EC 2.7.7.16), ribonuclease-free bacterial alkaline phosphatase (EC 3.1.3.1) and snake-venom phosphodiesterase (EC 3.1.4.1) were from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Scintillation chemicals 2,5-diphenyloxazole and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene were from Packard Instrument Co. (La Grange, Ill., U.S.A.). Triton X-100 was from Beckman Instruments Inc. (Fullerton, Calif., U.S.A.).

Methods

Preparation of mRNA. Rabbit globin mRNA was prepared from reticulocytes as described previously (Hunt, 1973). RNA samples were always checked for purity by polyacrylamide-gel electrophoresis on 2.4% polyacrylamide gels (Loening, 1967). The samples used here were more than 90% pure by this criterion. RNA concentration was determined by spectrophotometry assuming an $E_{260}^{1\text{cm}}$ value of 23 for 0.1% solutions in 0.1 M-sodium phosphate buffer, pH 7.0 (see Hunt, 1973).

Digestion with nucleases. *Pancreatic ribonuclease.* Digestion of Poly(U)–mRNA complexes by pancreatic ribonuclease at 10 $\mu\text{g}/\text{ml}$ was either in 0.1 M-NaCl, 0.001 M-Tris–HCl buffer, pH 7.6, or 0.6 M-NaCl, 0.001 M-tris–HCl buffer, pH 7.6, for 10 min at the stated temperature. Before centrifuging, sodium dodecyl sulphate was added to 0.1%. Alternatively, poly(A)·poly(U) complexes after digestion were pipetted on to Whatman 3MM paper at 0°C and washed in cold 5% (w/v) trichloroacetic acid (see below).

Spleen phosphodiesterase. mRNA (9.7 μg) was incubated with 0.23 units of spleen phosphodiesterase and 5 μg of bacterial alkaline phosphatase in 0.2 ml of 0.1 M-NaCl, 0.004 M-Tris–HCl buffer, pH 7.6, 0.004 M-MgCl₂ at 0°C for the stated times. After incubation sodium dodecyl sulphate to 0.1% and 10 μl (0.1 μCi) of ³H-labelled poly(U) were added before centrifuging. A unit of spleen phosphodiesterase activity is that amount of enzyme which hydrolyses 20 E_{260} units of RNA in 30 min at 37°C at pH 6.5.

Snake venom phosphodiesterase. mRNA (13.0 μg) was incubated with 250 μg of snake venom diesterase in 0.2 ml of 0.1 M-NaCl, 0.004 M-sodium borate buffer, pH 8.8, 0.004 M-MgCl₂ at 0°C for 8 h. After incubation 0.2 μCi of ³H-labelled poly(U) was added at 0°C followed by 2 μg of ribonuclease where indicated. Portions (0.1 ml) were pipetted on to Whatman 3MM paper and washed in cold 5% trichloroacetic acid (see below).

Sucrose gradients. Fractionation by centrifuging was on gradients (5.4 ml) of 5–20% (w/v) sucrose in either 0.1 M-NaCl, 0.001 M-Tris-HCl buffer, pH 7.6, or 0.6 M-NaCl, 0.001 M-Tris-HCl buffer, pH 7.6, in a Spinco SW 50.1 six-place swinging-bucket rotor in the Spinco model L2 ultracentrifuge at 4°C. After centrifuging the tubes were emptied by upward displacement through an Isco u.v.-light monitor. Fractions of 0.21 ml were collected.

Radioactivity counting. Samples (0.1 ml) were taken directly from fractionated gradients and mixed with 5 ml of a mixture of 0.5% 2,5-diphenyloxazole and 0.5% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene (2 vol.) and Triton X-100 (1 vol.). Efficiency of radioactivity counting of ^3H was 20% and of ^{14}C was 66% by using a Packard series 3000 Tri-Carb liquid-scintillation spectrometer. More usually polynucleotide samples, before or after nuclease treatment with 10 μg of ribonuclease/ml were pipetted on to strips (1 cm $\times$ 2 cm) of Whatman 3MM paper which were immediately immersed in 5% trichloroacetic acid for 1 h at 0°C. The papers were washed

three times for 10 min in 5% trichloroacetic acid at 0°C, twice in ethanol at 0°C, and twice in diethyl ether at room temperature, dried and counted by suspending in 10 ml of the toluene scintillation mixture omitting the Triton X-100. Radioactivity counting efficiencies of 20% for ^3H and 86% for ^{14}C were obtained.

Results

Interaction of poly(A) and poly(U) with mRNA

The first experiments were performed in 0.1 M-NaCl, 0.001 M-Tris-HCl buffer, pH 7.6, containing 0.1% sodium dodecyl sulphate. The mixture of mRNA and ^3H -labelled poly(U) was heated at 75°C for 10 min to melt any poly(A)·poly(U) double helixes and either cooled slowly at 50°C for 2 h or cooled fast in ice. Binding of poly(U) was calculated from radioactivity in the mRNA peak after sucrose-gradient centrifugation. No difference was found in the uptake of poly(U) in these experiments or when the mRNA and poly(U) were mixed at 0°C (Fig. 1

Fig. 1. Sucrose gradients of polynucleotide-mRNA mixtures in 0.1 M-NaCl

(a) ^3H -labelled poly(U) (0.1 μCi) and mRNA (5.9 μg) in a volume of 0.2 ml. (b) ^{14}C -labelled poly(A) (0.05 μCi) and mRNA (5.9 μg) in a volume of 0.2 ml. The direction of sedimentation was from left to right, and was at 37000 rev./min for 16.5 h. —, E_{254} ; ●, ^3H or ^{14}C radioactivity.

Table 1. Amount of poly(U) hybridized with mRNA under different conditions, as determined by sucrose-gradient sedimentation

In Expt. (a) the RNA used was Preparation 1 (14.8 μ g) in lines (i) and (ii) and Preparation 1 (5.9 μ g) in lines (iii) and (iv). The mixtures of RNA and poly(U) were in 0.1 M-NaCl, 0.001 M-Tris-HCl buffer, pH 7.6, containing 0.1 % sodium dodecyl sulphate. In Expt. (b) the RNA used was Preparation 2 (24.3 μ g) in line (i) and Preparation 2 (9.7 μ g) in lines (ii)–(vi). The mixtures of RNA and poly(U) were in 0.1 M-NaCl, 0.001 M-Tris-HCl buffer, pH 7.6. In Expt. (c) the RNA used was Preparation 2 (9.7 μ g). This experiment was a comparison of incubation of poly(U) and mRNA mixtures in 0.1 M-NaCl and 0.6 M-NaCl. In each experiment the volume of the reaction mixture was 0.2 ml. In all experiments mRNA–poly(U) preparations were assayed after centrifuging on 5–20 % sucrose gradients. Except in Expt. (c) lines (iii) and (iv) the preparations were all in 0.1 M-NaCl, 0.001 M-Tris-HCl buffer, pH 7.6. In Expt. (c) lines (iii) and (iv) they were in 0.6 M-NaCl, 0.001 M-Tris-HCl buffer, pH 7.6. Centrifugation at 4°C for Expt. (a) was at 37000 rev./min for 16.5 h; for Expt. (b), lines (i)–(iii) and Expt. (c), lines (i)–(iv) it was at 35000 rev./min for 16 h, and for Expt. (b) lines (iv)–(vi) it was at 38000 rev./min for 14 h.

Expt. (a) Treatment before centrifuging	Poly(U)/mRNA ratio	Poly(U) bound (%)	
(i) 75°C for 10 min, cooled fast	0.54	6.9	
(ii) 75°C for 10 min, cooled for 2 h at 50°C	0.54	5.4	
(iii) 75°C for 10 min, cooled fast	0.67	6.5	
(iv) Kept at 0°C	0.67	6.6	
Expt. (b) Treatment before centrifuging	Poly(U)/mRNA ratio	Poly(U) bound (%)	
Treatment of sucrose-gradient fractions ...		No ribonuclease	Ribonuclease for 10 min in 0.1 M-NaCl at 0°C
(i) Kept at 0°C	0.33	11.5	9.1
(ii) Kept at 0°C	0.41	12.2	5.0
(iii) Ribonuclease for 10 min at 0°C	0.41	6.7	4.0
(iv) Kept at 0°C	0.41	11.1	4.1
(v) Spleen diesterase for 4 h at 0°C	0.41	18.6	7.7
(vi) Spleen diesterase for 8 h at 0°C	0.41	17.7	7.6
Expt. (c) Treatment before centrifuging	Poly(U) bound (%)		
Treatment of sucrose-gradient fractions ...	No ribonuclease	Ribonuclease for 10 min in 0.6 M-NaCl at 37°C	
(i) Kept at 0°C in 0.1 M-NaCl	12.2	9.5	
(ii) Ribonuclease for 10 min at 0°C in 0.1 M-NaCl	6.8	6.0	
(iii) Kept at 0°C in 0.6 M-NaCl	Aggregation	Aggregation	
(iv) Ribonuclease for 10 min at 37°C in 0.6 M-NaCl	12.2	7.4	

and Table 1, Expt. a). When 14 C-labelled poly(A) was incubated with mRNA at 75°C for 10 min and cooled fast in ice no uptake of poly(A) occurred (Fig. 1). In the experiments in Table 1, Expt. (a) where mRNA and mRNA–poly(U) complexes were sedimented under identical conditions the relative *s* value of the mRNA–poly(U) complex was 10.6S compared with mRNA alone at 10.0S. This difference was only significant at the 10 % level.

In subsequent experiments *s* values were calculated assuming that the mRNA–poly(U) complex sediments at 10.6S (Table 2).

Ribonuclease treatment of the poly(U) hybrids

Pancreatic ribonuclease treatment of the poly(U)–mRNA hybrids isolated by sucrose-gradient centrifugation completely degraded the hybrid when incubation was at 37°C in 0.1 M-NaCl. Incubation of

poly(U)·poly(A) hybrids in 0.1M-NaCl with ribonuclease showed that 50–75% of the poly(U) in the complex was stable at 0°C but not at higher temperatures (Table 3, Expt. a). By contrast the complex was completely stable at 37°C in 0.6M-NaCl (Table 3, Expt. b).

A second preparation of mRNA was hybridized with poly(U) and subsequently digested with pancreatic ribonuclease for 10min at 0°C in 0.1M-NaCl before centrifuging on a sucrose gradient. Under these conditions a hybrid peak with a sedimentation coefficient of 4.6S was obtained (Fig. 2, Table 2). Estimates of the amount of poly(U) bound under these conditions before and after ribonuclease treatment, and before and after ribonuclease treatment of the sucrose-gradient fractions at 0°C for 10min are given in Table 1, Expt. (b). The higher binding of poly(U) found in the preparation may be explained by the low E_{260}/E_{280} ratio (<2) found in the first mRNA preparation which would result in an over-estimation of the RNA concentration. The second sample of mRNA had an E_{260}/E_{280} ratio >2 which is expected for pure RNA.

Since the treatment with pancreatic ribonuclease at 0°C in 0.1M-NaCl produced somewhat variable results the alternative conditions of digestion, 37°C in 0.6M-NaCl, were used. Under these conditions of ribonuclease digestion after sucrose-gradient fractionation the decrease in bound poly(U) is lower for the hybrid formed in 0.1M-NaCl at 0°C and there is only a 12% decrease in binding when the hybrid is

digested at 0°C in 0.1M-NaCl before centrifuging (Table 1, Expt. c, lines i and ii).

Digestion with exonucleases

mRNA was digested with both snake venom phosphodiesterase and spleen phosphodiesterase, exonucleases attacking from the 3' and 5' ends respectively, before hybridizing with poly(U) and

Table 2. *Sedimentation coefficients for various polynucleotides*

All results were calculated from relative sedimentation in sucrose gradients assuming that the mRNA–poly(U) hybrid has an s value of 10.6 (see the text for discussion). * Shows s.d. from replicates within experiments; other s.d. values were between experiments. Correction to $s_{20,w}$ values were made by using the method of McEwen (1967).

Polynucleotides	$s_{20,w}$ (S)
Poly(U)	2.28 ± 0.12
Poly(A)	4.75 ± 0.11
Poly(A)·poly(U)	8.3
mRNA	10.0 ± 0.09*
mRNA–poly(U)	10.6 ± 0.23*
mRNA–poly(U) (after ribonuclease)	4.59 ± 0.17

Table 3. *Stability of the poly(A)·poly(U) hybrid to ribonuclease digestion*

Equimolar amounts of poly(A) and ^3H -labelled poly(U) were mixed at 0°C in 0.1M-NaCl, 0.001M-Tris–HCl buffer, pH 7.6, or 0.6M-NaCl, 0.001M-Tris–HCl buffer, pH 7.6, and then incubated for 10min with 10μg of pancreatic ribonuclease/ml; polymer concentration was 20μg/ml.

Expt. (a) Incubation temperature (°C)

	Degradation of poly(U) in 0.1 M-NaCl (%)	
	With poly(A)	Without poly(A)
0	43	99
9.5	86	99
21.0	100	100
28.5	100	100

Expt. (b) Incubation temperature (°C)

	Degradation of poly(U) (%)		
	0.1 M-NaCl; with poly(A)	0.6 M-NaCl	
		With poly(A)	Without poly(A)
0	24.2	0	81
37	—	0	100

Fig. 2. Sucrose gradients of ribonuclease digests of poly(U)-mRNA hybrids in 0.1 M-NaCl

(a) and (b) ^3H -labelled poly(U) ($0.1 \mu\text{Ci}$) and mRNA ($9.7 \mu\text{g}$) in a volume of 0.2 ml. A portion (0.1 ml) from each fraction was precipitated on paper in (a), the remainder (0.1 ml) was digested with $1 \mu\text{g}$ of ribonuclease for 10 min at 0°C before precipitation of 0.1 ml portions (b). (c) ^3H -labelled poly(U) ($0.1 \mu\text{Ci}$) and mRNA ($9.7 \mu\text{g}$) in a volume of 0.2 ml were incubated with $2 \mu\text{g}$ of ribonuclease before centrifuging. Portions (0.1 ml) from each fraction were precipitated on paper for counting of radioactivity; the direction of sedimentation was from left to right, and was at 35000 rev./min for 16 h. —, E_{254} ; ●, ^3H radioactivity.

Table 4. Amount of poly(U) hybridized with mRNA after snake venom diesterase digestion

Conditions of incubation are as described in the text.

Treatment	Poly(U) bound (%)
(i) None	12.9
(ii) Snake venom phosphodiesterase for 8 h at 0°C	4.8
(iii) mRNA omitted	0.3

centrifuging. Unfortunately, the snake venom phosphodiesterase digests formed aggregates and could not be assayed on the sucrose gradients. Treatment with spleen phosphodiesterase for 4 and 8 h at 0°C caused breakdown of mRNA to heterodisperse material sedimenting with a mean *s* value of 6S. However hybridization with poly(U) was not diminished (Table 1, Expt. b, lines iv–vi). This indicated that no poly(A) sequences were lost by digestion of mRNA from its 5' end by this exonuclease.

To overcome the problem of aggregation found under the conditions of incubation with snake venom diesterase, the amount of poly(U) bound to mRNA was assayed by precipitation on to Whatman 3MM filter paper and washing with 5% trichloroacetic acid after further digestion with pancreatic ribonuclease at 0°C. The results shown in Table 4 indicate that after treatment for 8 h a decrease of 64% in the amount of hybridization occurs. This represents hybridization equivalent to 5% binding, which is not very different from the value of 6.4% found from sucrose gradients. This indicates that there is a decrease in the number of available ends after digestion with snake venom diesterase, which together with the absence of any effect by spleen enzyme would locate the poly(A) regions at the 3' terminus of mRNA.

Discussion

Previous studies on the interaction of homopoly-nucleotides with RNA by Hayes *et al.* (1966) showed no interaction of poly(A) or poly(U) with *Escherichia coli* rRNA in 0.1M-NaCl. However, a substantial binding occurred in 0.6M-NaCl or in 0.1M-NaCl in the presence of divalent cations. The results reported here show a strong interaction between mRNA and poly(U) in 0.1M-NaCl but no corresponding interaction of poly(A) with either native or heat-denatured mRNA. The interaction between poly(U) and mRNA is not changed after heating the mRNA at 75°C in 0.1M-NaCl, conditions which would melt any

poly(A)·poly(U) double helices. It must therefore be assumed that the poly(A) sequences detected at 0°C are the only ones present in the mRNA.

Since poly(U), when hybridized in excess with poly(A) forms a poly(A)·2poly(U) triple helix, any estimation of the amount of poly(A) in RNA would depend on whether a double or triple helix is being formed. The amount of poly(U) hybridized with mRNA in the absence of ribonuclease is $11.7\% \pm 0.47\%$. Assuming that 10S mRNA has a molecular weight of approx. 170000 or a chain length of 500 nucleotides, this percentage corresponds to 58 ± 2 nucleotides. The molecular weight of the poly(U) in these experiments was calculated, by using data from Fresco & Doty (1957) and Richards *et al.* (1963), and an $s_{20,w}$ value of 2.28S (Table 2), as 23500 or 70 nucleotides. It may then be assumed that either all of the mRNA molecules have poly(A) sequences sufficient to hybridize or partially hybridize 1 molecule of poly(U) as a double helix, or that a fraction of the molecules hybridize with more than one poly(U). The fact that the poly(U)–mRNA hybrid shows a shift in *s* value and constant specific radioactivity over the peak on a sucrose gradient would favour the former interpretation. The change of molecular weight from 190000 to 215000 calculated from *s* values of 10S and 10.6S is also consistent with this interpretation.

A poly(A)·2poly(U) triple helix is as stable as a poly(A)·poly(U) double helix in 0.6M-NaCl, but is less stable than the double helix in 0.1M-NaCl (Blake & Fresco, 1966; Massoulié, 1968). The stability of octadecamer hybrids is much lower than hybrids from longer oligonucleotides (Pörschke, 1971). It could be interpreted from these facts that the amount of hybridization in 0.6M-NaCl after digestion at 37°C (10.8%) represents triple-helix formation and the hybridization in 0.1M-NaCl after digestion at 0°C (6.4%) represents double-helix formation. In any case the amount of binding found would indicate that 30–40 nucleotides in the mRNA are in a poly(A) region. This figure is in agreement with the value of 50–70 nucleotides found by Lim & Canellakis (1970) in A-rich regions in rabbit globin mRNA.

These results with the terminal-sequence analysis reported in the preceding paper (Hunt, 1973) indicate that the polyadenylate regions in rabbit globin mRNA are present at the 3' termini of both α - and β -globin mRNAs and that although they are rich in adenylate other nucleotides are interspersed in the sequences in a specific manner.

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References

- Blake, R. D. & Fresco, J. R. (1966) *J. Mol. Biol.* **19**, 145–160
- Burr, H. & Lingrel, J. B. (1971) *Nature (London) New Biol.* **233**, 41–43
- Fresco, J. & Doty, P. (1957) *J. Amer. Chem. Soc.* **79**, 3928
- Hayes, D. H., Grunberg-Manago, M. & Guérin, M. F. (1966) *J. Mol. Biol.* **18**, 477–498
- Hunt, J. A. (1973) *Biochem. J.* **131**, 315–325
- Lim, L. & Canellakis, E. S. (1970) *Nature (London)* **227**, 710–712
- Loening, U. E. (1967) *Biochem. J.* **102**, 251–257
- Massoulié, J. (1968) *Eur. J. Biochem.* **3**, 428–438
- McEwen, C. R. (1967) *Anal. Biochem.* **20**, 114–149
- Pörschke, D. (1971) *Biopolymers* **10**, 1989–2013
- Richards, E. G., Flessel, C. P. & Fresco, J. R. (1963) *Biopolymers* **1**, 431–446