Calf lens crystallin messenger RNA's contain polynucleotide sequences rich in adenylic acid

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ABSTRACT

Calf lens messenger fractions have been purified on poly(dT)-cellulose. The purified RNA's have been analyzed as nucleosides by a method which can be used on a micro-scale with highly reproducible results. Both the 10S and 14S lens mRNA contain poly A sequences. The poly A content found in 14S mRNA cannot account for the excess nucleotides in this message.

INTRODUCTION

The presence of polynucleotide sequences rich in adenine has been recognized as a common feature of eukaryotic messenger RNA's, with the notable exception of the messengers for the histones (1). The 10S and 14S RNA fractions from calf lens, which direct the synthesis of crystallin polypeptides (2, 3), seemed to have base compositions with rather low adenine content (4) suggesting the possible absence of long poly A sequences. To detect these particular sequences we have further purified the 10S and 14S RNA fractions by column chromatography on cellulose containing covalently linked poly (dT) chains (average size 60 nucleotides). The purified RNA has been analyzed to determine its base composition by digestion to the level of nucleosides and by chromatographic separation of the nucleosides; the method adopted can be used in the microgram scale with excellent results. The affinity for the poly(dT)-cellulose column and the adenine enrichment in the isolated fractions demonstrate that the calf lens messenger RNA's contain poly A sequences. In particular the base composition analysis of the 14S mRNA purified by poly (dT) cellulose and sucrose gradient centrifugation shows that this RNA has a GC content which is much higher than the value found for the total DNA of calf.

MATERIAL AND METHODS

All chemicals were analytical grade. Alkaline phosphatase (E.C.3.1.3.1.) was obtained from Worthington (Freehold, N.J.). Pancreatic RNAase and T₁ RNAase were obtained from Sigma Chemical Co. 18S + 28S calf lens ribosomal RNA was purified as previously described (4). RNA of tobacco mosaic virus (TMV-RNA) was a gift of Dr. H. Guillely and M₁₂ RNA was kindly provided by Dr. R.N.H. Konings.

Chromatography of 10S and 14S RNA fractions on poly (dT)-cellulose

10S and 14S RNA fractions were extracted from calf lens polysomes and isolated by zonal centrifugation as described previously (4). These materials were further purified on a column of poly(dT)-cellulose prepared as follows: 100 mg of oligo(dT)-cellulose (5) were converted into poly(dT)-cellulose by extending the short oligo(dT) chains with additional thymidylate units (to approximate length of 50-70 nucleotides) by the terminal deoxynucleotidyl transferase reaction as previously described (6). The column (0.3 x 2 cm) was washed with 0.1 M NaOH and equilibrated with 0.01 M Tris-HCl pH 7.5-0.5 M KCl or with 0.01 M Tris-HCl pH 7.5-0.1 M NaCl-0.05% sodium dodecylsulfate (SDS) (buffer A). Elution of the lens RNA's was started with the same buffer and a constant flow rate was maintained with a peristaltic pump (Technicon, LTD, Dublin, Ireland). The ultraviolet absorbancy of the column effluent was monitored using a Uvicord (LKB, Stockholm, Sweden) equipped with a flow cell of 0.1 cm optical path. After the complete elution of the large peak of non-retained material a washing with 0.01 M Tris-HCl pH 7.5 or 0.001 M Tris-HCl pH 7.5-0.05% SDS (buffer B) was started and the temperature was raised to the values indicated in the legends of figures 1 and 4 and Tables III and IV, resulting in the elution of the remaining material.

Centrifugation of the 14S RNA on sucrose gradient

The product eluted at 50° from the poly (dT)-cellulose column was applied directly on a 10 to 35% isokinetic sucrose gradient (7) containing 0.01 M Tris-HCl pH 7.5 - 0.05 M NaCl and centrifuged in a SW 41 Spinco rotor for 14 h at 5°.

Base composition determination

0.08 - 0.12 A₂₆₀ units of RNA samples were either dialyzed against H₂O or precipitated with ethanol to remove excess salts, prior to drying under reduced pressure. The products were dissolved in 10 µl each of 0.33 M NaOH

and placed in glass capillary tubes. The tubes were sealed and the RNA was hydrolyzed by heating at 80° for 40 min. Under these conditions quantitative hydrolysis of the RNA is achieved with no apparent loss of nucleosides (8). After the incubation, the tubes were immediately opened and the pH was adjusted near a value of 9 by addition of 10 μ l of 0.33 M HCOOH and 5 μ l of 1 M ammonium carbonate. Dephosphorylation of nucleotides was carried out with 1 μ l of alkaline phosphatase 40 units/ml at 37° for 16 h. Nucleoside analysis was usually made on 10 μ l of the reaction mixture which was concentrated to dryness, then dissolved in 5 μ l of 0.4 M ammonium formate pH 4.65 and loaded on a column (0.2 x 9 cm) of Aminex A-6 (BioRad, Richmond, Calif.) equilibrated with the same solvent (8, 9); nucleoside elution peaks were detected as already described (10) and a new method for transmission recording and peak area integration was used (11).

For the calculation of the percentage of each nucleoside in the analyzed mixture, peak areas were multiplied by a factor proportional to the molar absorption of the nucleosides. These factors were calculated at 271 nm as previously described for the deoxynucleosides (12) and are equal to 0.8986; 1.036; 0.9954; 1.078 for U, G, A and C, respectively.

The nature of the U.V. absorbing material eluted before U was investigated by loading the column with a blank processed through the whole sequence of operations done for the RNA samples. The chromatogram obtained showed U.V. absorbing peaks which were identical in elution volume, amount and profile to those obtained in the nucleoside analysis.

The reliability of the method has been tested on an artificial mixture of ribonucleosides, tobacco mosaic virus RNA and M12 phage RNA (Table I). The base composition values found for the two RNA's were in excellent agreement with those reported by other investigators (13, 14).

Analytical centrifugation

Analytical centrifugation was carried out in a Spinco model E instrument equipped with a monochromator and a photo-electric scanner. Band sedimentation was performed in Vinograd 30 mm K₂SO₄ cells. 25 μ l aliquots of the samples having an $A_{260} = 0.6$ were layered on 1 M NaCl - 0.01 M Tris-HCl pH 7.6 and centrifuged at 36,000 rpm. Scannings were done at 265 nm; the horizontal enlargement of the scannings was equal to 23.4.

RESULTS AND DISCUSSION

The fractions of 10S and 14S RNA's from calf lens obtained after the zonal

centrifugation were analyzed to determine their base composition.

Table II shows that two different preparations of each RNA have almost identical base composition. The values obtained on these samples with the new method of analysis, though greatly differing from those previously published (4), again show the presence of a low percentage of adenine, that is, the opposite of what is expected for mRNA molecules having long sequences of poly A.

To determine whether our RNA preparations consisted of several different RNA's, we chromatographed the 10S RNA fractions on a column of poly(dT)-cellulose as described in Materials and Methods and in legend of fig. 1, where a typical chromatogram is shown.

In fact the column chromatography showed that the RNA preparations were not homogeneous and at least three classes of molecules can be resolved. Furthermore the chromatographic behavior and the base composition of the fractions demonstrated that these may differ in their primary and secondary structures. Fig. 2 shows the nucleoside chromatographic pattern from two different fractions of 10S RNA and Table III summarizes the results of the base analysis done on the 10S RNA after chromatography on poly(dT)-cellulose. Table III shows the data obtained for the fraction not retained by the column, the fractions eluted at 45°, and at 60°, as well as the percentage represented by each fraction. The base composition of a sample of ribosomal RNA 18 + 28S from calf lens is also shown for comparison.

The base composition of RNA not retained by the poly(dT)-cellulose was very similar to that of ribosomal RNA, thus suggesting that the unbound RNA was mainly composed of fragments of ribosomal RNA. The RNA eluted by lowering the ionic strength and raising the temperature to 45° had a base composition nearly equal to that of the not-retained material. This RNA might be composed of both ribosomal RNA fragments with an extent of secondary structure differing from that of unbound RNA and mRNA molecules with short poly A segments. Finally, a great enrichment in adenine was observed in the fraction eluted at 60°. The high affinity for the poly(dT)-cellulose column and the base composition of these fractions strongly suggest the presence of poly A sequences. In order to confirm the presence of these sequences we have treated the 10S RNA eluted at 60° with pancreatic and T₁ RNAases to detect RNAase resistant homopoly-nucleotides like poly A (15). The digest was loaded on the poly(dT)-cellulose column and chromatographed as previously described: 20% of

Fig. 1. Chromatography on poly(dT)-cellulose column of 10S RNA. 5.2 A_{260} units of 10S RNA from calf lens were loaded on the column in 0.5 ml of 0.01 M Tris-HCl pH 7.5 - 0.5 M KCl. The column was eluted with the same buffer at a flow rate of 0.8 ml/hr. After the complete elution of the large peak of not-retained material, the eluting buffer was changed to 0.01 M Tris-HCl pH 7.5 and the temperature of the column was raised to 45° and finally to 60° as indicated by the arrows.

Fig. 2. Chromatography on a Aminex A-6 column of ribonucleosides obtained by digestion of a) 10S RNA not-retained by the poly(dT)-cellulose column and b) 10S RNA eluted at 60°. The flow rate of the eluting buffer was 0.75 ml/hr.

Fig. 3. Photoelectric scanning profiles obtained by the analytical ultracentrifuge. Band sedimentation of a) 10S RNA not-retained by the poly(dT)-cellulose column and b) 10S RNA eluted at 60°. The interval of time between the two scanings is equal to 8 min. in both experiments. The arrow indicates the direction of sedimentation.

Fig. 4. Chromatography on poly(dT)-cellulose of 14S RNA. 4.5 A_{260} units of 14S calf lens mRNA, obtained from zonal centrifugation, in 0.2 ml of 0.01 M Tris-HCl, pH 7.5 - 0.1 M NaCl - 0.05% SDS, were loaded on the column. Elution was performed as described in Materials and Methods. The column was maintained at 25° for the first 60 min. of elution and at 50° thereafter.

Table I

Analytical results obtained with an artificial mixture of ribonucleosides, Tobacco Mosaic Virus RNA and M₁₂ phage RNA.

Nucleoside	Artificial mixture of known composition		TMV RNA		M ₁₂ RNA	
	% Loaded	% Found	% Found	Published (13)	% Found	Published (14)
U	36.5	36.8 $\pm$ 0.9	27.2 $\pm$ 0.1	27.7	23.7 $\pm$ 1.0	24.5
G	13.5	13.1 $\pm$ 0.2	25.6 $\pm$ 0.1	24.9	28.5 $\pm$ 0.2	26.8
A	36.4	36.5 $\pm$ 0.2	27.4 $\pm$ 0.5	28.2	21.5 $\pm$ 0.2	23.1
C	13.6	13.6 $\pm$ 0.2	19.7 $\pm$ 0.7	19.2	26.2 $\pm$ 0.5	25.6

The values represent the molar fraction of each nucleoside and are the average of at least 2 determinations.

Table II

* Base composition of 10S and 14S RNA fractions from calf lens polysomes

Nucleoside	10S RNA			14S RNA		
	Published Results(4)	Present Work		Published Results(4)	Present Work	
	**	I Prepar.	II Prepar.	**	I Prepar.	II Prepar.
U	30.3	19.1 $\pm$ 0.3	20.8	30.4	19.1 $\pm$ 0.4	21.6
G	28.9	29.8 $\pm$ 0.3	29.2	30.4	29.9 $\pm$ 0.6	30.3
A	18.9	24.0 $\pm$ 0.3	25.0	19.2	22.9 $\pm$ 0.4	21.9
C	21.9	27.1 $\pm$ 0.8	25.1	20.0	28.2 $\pm$ 1.1	26.1

* The values represent the molar fraction of each nucleoside.

** Average of 4 determinations.

Table III

*Base composition of 10S fractions eluted from the poly(dT)-cellulose column

Nucleoside	Ribosomal RNA	** RNA not-retained 82%	RNA eluted at 45 ⁰ 4%	RNA eluted at 60 ⁰ 14%
U	18.5 ± 0.5	20.5 ± 0.1	20.6	17.6
G	33.6 ± 0.9	30.6 ± 0.1	29.3	24.7
A	18.2 ± 0.1	20.6 ± 0.2	22.1	35.1
C	29.5 ± 0.5	28.2 ± 0.0	28.0	22.6

* The values represent the molar fraction of each nucleoside.

** Average of two determinations.

the product was retained on the column and could be eluted by raising the temperature to 60°. The analysis showed the presence of excess adenine in this product.

The amount of adenine found in the fractions eluted at 60° is strikingly high with respect to the starting material. For this reason a control experiment was carried out to determine if any reduction of molecular weight has occurred during the chromatography on poly(dT)-cellulose, since a slight degradation could cause enrichment in poly(A) sequences of these fractions.

We determined the sedimentation coefficient by band sedimentation in the analytical ultracentrifuge after the poly(dT)-cellulose chromatography of two fractions of 10S RNA (see fig. 3). A $s_{20,w}$ value of 11S was found for both fractions. In the case of 10S RNA eluted at 60° a slight dissymmetry in the sedimenting peak suggests the presence of molecules of lower sedimentation coefficient (fig. 3b) which could be responsible for a possible overestimation of adenine in this fraction.

To avoid any artefact resulting from unwanted degradation of the messenger, we have slightly modified the poly(dT)-cellulose chromatographic procedure and have further purified the adenine rich RNA fraction on a sucrose gradient, in order to remove slow sedimenting poly A containing fragments. In this case the work has been done on the calf lens 14S RNA, which directs the synthesis of a single polypeptide, the A₂ chain of

Table IV

*Base composition of 14S chromatographical fractions eluted from the poly(dT)-cellulose column

Nucleoside	Starting material 14S RNA	RNA non-retained loaded in buffer A**	RNA eluted at 25°		RNA eluted at 50°	
			with buffer B**		with buffer B** and centrifuged in sucrose gradient	
		84 %	6 %		10 %	
U	21.0 ± 0.5	20.5 ± 0.5	19.8 ± 0.1		17.5 ± 0.1***	
G	31.0 ± 0.7	32.0 ± 1.0	30.4 ± 0.2		26.8 ± 0.1	
A	21.3 ± 0.6	21.4 ± 0.5	22.8 ± 0.4		26.8 ± 0.1	
C	26.5 ± 0.5	26.0 ± 1.0	27.2 ± 0.5		28.8 ± 0.1	

* The values represent the molar fraction of each nucleoside and are the average of at least two determinations.

** See Materials and Methods.

*** The analysis were done on pooled fractions from the sucrose gradient containing 14S RNA.

α crystallin and for which the possible presence of long poly A untranslated regions may be postulated to explain the excessive length of the messenger (16). The RNA obtained from zonal centrifugation was loaded on the poly(dT)-cellulose column in 0.01 M Tris-HCl pH 7.5 - 0.1 M NaCl - 0.05% SDS and the subsequent elution at low ionic strength with 0.001 M Tris-HCl pH 7.5 - 0.05% SDS was first done at 25⁰ and later at 50⁰ (fig. 4). The fraction eluted at high temperature was further purified on an isokinetic sucrose gradient (see Materials and Methods) and the material eluting as a single component in the 14S position pooled and analyzed.

Table IV shows the percentages of recovery of each fraction and the base composition determination. The results are similar to those described in table III except that the fraction eluting at 50⁰ and further purified on the sucrose gradient was characterized by an enrichment in adenine of only 5 - 6% with respect to the starting material. Indeed the base composition of 14S RNA isolated by this method is probably that of a homogeneous mRNA. Two features are evident: the adenine content is not exceedingly high and cannot by itself account for the discrepancy between the messenger and the polypeptide chain length. The GC content of 55% is much higher than the value of 44% found for the total DNA of calf (17).

In conclusion the affinity for the poly(dT)-cellulose and the base composition analysis demonstrate the presence of poly A sequences in 10S and 14S mRNA's. A similar result has been found for nearly all eukaryotic mRNA's. This sequence allows the use of these RNA's as a template for the reverse transcriptase reaction using oligo(dT) as primer (18, 19).

The use of poly(dT)-cellulose columns is very promising for the purification of messenger RNAs having molecular weight close to ribosomal RNAs. In fact it allows the formation of stable hybrids, which is not the case for the oligo(dT)-cellulose (20) and permits the selection of RNA molecules characterized by the presence of long poly A sequences.

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