Lack of poly(A) sequence in half of the messenger RNA coding for ewe α_S casein

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SUMMARY

Poly(U)-sepharose chromatography of RNA from bound polysomes of lactating ewe mammary gland results in the isolation of an RNA fraction containing mRNA for α_S casein. Several independent experimental approaches revealed the existence of poly(A) sequences the average length of which was 130 nucleotides. Only about 40% of the messenger activity for α_S casein has a poly(A) sequence.

INTRODUCTION

An RNA fraction containing mRNA coding for α_S casein has been previously isolated in this laboratory from lactating ewe mammary gland by sucrose gradient centrifugation[1].

Recent studies indicate that most of the mRNAs of mammalian cells contain a large poly(A) segment[2,3]. In this paper we present evidence for the existence of poly(A) in the ewe mammary gland mRNAs. The mean length of poly(A) was estimated : 1°) by polyacrylamide gel electrophoresis of mRNA hydrolyzed by T_1 and pancreatic RNAses ; 2°) from the nucleotide composition of mRNA ; 3°) from measurements of the fluorescence of mRNA-poly(U)-ethidium bromide complex. The quantification of messenger activity in a cell-free system of polysomal RNAs hybridizable and not hybridizable to poly(U)-sepharose revealed that no more than half of the messenger molecules have a poly(A) sequence.

MATERIALS AND METHODS

1) Preparation of membrane-bound polysomes and RNA.

Post mitochondrial supernatant was centrifuged at 18 000 rpm for 15 min. The microsomal pellet was resuspended in

TKM buffer* containing 50 µg/ml heparin, 3 % Triton X 100, 0,25 % DOC* and centrifuged at 25 000 rpm for 5 h over 8 ml 1 M sucrose.

RNA was extracted from polysomes and microsomes with SDS-phenol at pH 9 according to LEE et al.[2] or with SDS-phenol-chloroform at pH 9.

2) Poly(U)-sepharose chromatography.

Poly(U) was bound to sepharose according to WAGNER et al.[4].

Total polysomal RNA was applied to the column in TNES buffer* as described by FIRTEL et al.[5] and eluted by 0.05 % SDS in water. The eluate was adjusted to 0.12 M NaCl and immediately rechromatographed on poly(U)-sepharose previously reequilibrated with TNES buffer. This second chromatography was necessary to obtain an RNA fraction devoid of rRNA. A similar degree of purity was obtained by a single poly(U)-sepharose chromatographic step when starting from mRNA enriched fraction 7-17 S prepared by zonal centrifugation (to be published).

3) Messenger activities were tested in two different translational systems :

- System I : Translation in a reticulocyte lysate. Preparation of the lysate was as described by LINGREL[22]. The assay mixture contained in a total volume of 250 µl : 100 µl of lysate, 80 mM KCl, 2 mM $(CH_3\ COO)_2$ Mg, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, creatine phosphokinase (15 units per ml), a mixture of amino-acids 100 µM minus Ileu and Pro, ^{14}C Ileu 0,5 µCi (Spec. Act. 150 mCi/m mole), ^{14}C Pro 0,5 µCi (Spec. Act. 150 mCi/m mole), hemin 20-30 µM, 2 µg of purified mRNA or 10-20 µg of other mRNA containing fractions. These two labelled aminoacids were choosen according to the respective aminoacid composition of casein and globins thus insuring the best incorporation of aminoacids in casein. All incubations were carried

* Abreviations used : - TKM buffer : Tris 50 mM pH 7.5, KCl 150 mM $MgCl_2$ 5 mM.
- DOC : sodium deoxycholate.
- TNES buffer : 0.01 M Tris pH 7.6, 0.12 M NaCl, 0.1mM EDTA, 0.5 % SDS.

out for 90 min. at 26°C. To 100 µl of the mixture were added at the end of incubation 5 µg of α_s-casein, 100 µl of anti-α_s-casein serum and phosphate buffer (10 mM sodium phosphate, 150 mM NaCl) containing Triton X 100 and sodium deoxycholate 0,5 % each at final concentration in a total volume of 250 µl. The immunoprecipitate was collected by centrifugation at 18 000 rpm over 1 ml 0.6 M sucrose in phosphate buffer containing the detergents[23]. This system was used with mammary mRNA when specific α_s-casein synthesis was measured.

- System II : Translation in a mRNA-dependent cell free system. This system was constituted of ribosomal sub-units prepared according to FALVEY and STAEHELIN[24], dialysed post ribosomal supernatant, $tRNA_s$ (all extracted from mammary gland) and initiation factors (extracted from reticulocytes ribosomes with 0.5 M KCl)[25]. The assay mixture contained in a total volume of 150 µl : Tris HCl pH 7,6, 20 mM ; KCl, 90 mM ; $MgCl_2$, 3.5 mM ; ATP, 1 mM ; GTP, 0.2 mM ; PEP, 3 mM ; pyruvate kinase, 0.1 IU ; DTT, 1 mM ; 19 ^{12}C aminoacids, 50 µM ; ^{14}C leucine, 0.5 µCi ; 0.18 A_{260nm} of tRNA ; post ribosomal supernatant, 300 µg of protein ; ribosomal sub-units, 0.6 A_{260nm} ; 0.5 M KCl wash fraction, 200 µg of protein. The incubation was carried out at 37°C for 45 min. The proteins synthesized by this procedure were recovered by adding 2 ml of 10 % TCA, heating to 90°C for 15 min. and collected over glass filter. This system was used with mammary and reticulocyte mRNA when total protein synthesis was measured.

4) Formamide polyacrylamide gel electrophoresis was conducted according to STAYNOV et al.[6].

5) Nucleotide composition was determined by thin layer chromatography on cellulose plate of RNA hydrolysed for 2 h at 100°C in 15 % piperidin.

6) The fluorescence method used to detect and quantify Poly(A) is described in the results and legend of figure 2. Fluorescence was measured with a Jobin-Yvon spectrofluorimeter. The cuvette containing 600 µl of solution was inserted in a cuvette holder thermostated at 25°C.

Synthetic poly(A) and poly(U) were from Miles, T_1 RNAse from Sankyo LTD. Ethidium bromide was a gift of Dr Cobb of the

Boots and Pure Drug Co. (Nottingham, England).

RESULTS

A - ISOLATION AND CHARACTERIZATION OF mRNA BY POLY(U)-SEPHAROSE CHROMATOGRAPHY

1) Poly(U)-sepharose chromatography

Poly(U)-sepharose chromatography led to the isolation of an RNA fraction corresponding to 0.8 - 1 % of total polysomal RNA applied to the column.

Table I

QUANTIFICATION OF MESSENGER ACTIVITY FOR α_s CASEIN HYBRIDIZABLE TO POLY(U)-SEPHAROSE

RNA extraction	RNA applied to poly(U)-seph.	% RNA retained by poly(U)-seph.	% messenger activity for α_s casein (1)	% cpm TCA precipitable (2)
SDS	7-17 S SW 27	12	30	
Phénol	7-17 S	14	38	35
SDS	Zonal	18	58	
	Centrifugation	16.5	60	
SDS Phenol	Total Polysomal RNA	0.9	50	
SDS* Phenol	7-17 S Zonal Centrifugation	18		70

* This fraction was obtained from rabbit reticulocytes

(1) Translation in system I [see METHODS (3)]

(2) Translation in system II ["]

This RNA fraction contained messenger activity for several milk proteins and particularly for α_s casein[8] (to be published). The messenger activity per A_{260} unit was 40 times higher in the RNA fraction hybridizable to poly(U)-sepharose than in total polysomal RNA.

Measurement of messenger activity for α_s casein in the effluent and eluate of the column indicated that no more than 40 % of messenger activity could be hybridized to poly(U)-sepharose.

A similar result was obtained by chromatography of 7-17 S RNA fraction isolated by zonal centrifugation from polysomal RNA extracted by SDS-phenol or SDS alone just before being applied to the gradient.

An identical proportion was obtained for the total messenger activity in a mRNA-dependent cell-free system by TCA precipitation of synthesized total protein.

With rabbit reticulocytes the same experiment indicated that 70 % of total messenger activity was retained by the column.

These results summarized in table I suggest that for at least half of the mRNAs, the poly(A) sequence is non-existent, too short to form a stable hybrid with poly(U) or masked in mRNA structure.

2) Estimation of the loss of poly(A) in mRNA after phenol extraction.

In order to check that an important cleavage of poly(A) from mRNA was not due to phenol extraction, two control experiments were done.

1. A known amount (6 A_{260}) of mammary messenger containing poly(A), previously purified by poly(U)-sepharose column, was added to mammary polysomes (50 A_{260}). This mixture was extracted as described in METHODS at pH 9 with SDS-phenol. The total RNA obtained was chromatographed on poly(U)-sepharose. The 6 A_{260} mRNA added to polysomes were quantitatively recovered in the eluate of the column.

2. Total mammary RNAs were labelled in monolayer cell culture by addition of ^3H uridine to the medium. After 24 h of labelling total cell nucleic acids were extracted by phenol-SDS chloroform at pH 9. A known amount (4.10^6 cpm) of this labelled

RNA was chromatographed twice successively (see METHODS) on poly(U)-sepharose, directly or after having been reextracted by phenol-SDS-chloroform at pH 9 in the presence of mammary polysomes (50 A_{260}) or mammary tissue (1 g). The amount of labelled RNA hybridized to poly(U)-sepharose was 0.33 %, 0.33 % and 0.36 % respectively of the initial radioactivity. (When total nucleic acid from mammary gland was chromatographed on poly(U)-sepharose, about 0.2 - 0.3 % of the optical density was hybridized to the column).

These two experiments suggest that a cleavage of poly(A) in half of the mRNA molecules during phenol extraction is unlikely.

B - CHARACTERIZATION OF POLY(A) SEQUENCE

1) Identification of poly(A) sequence by electrophoresis

The 7-17 S fraction of zonal centrifugation was submitted to poly(U)-sepharose chromatography. Both effluent and eluate were hydrolyzed by a mixture of T_1 and pancreatic RNAses[2] for two hours. The hydrolysates were chromatographed on poly(U)-sepharose. Only the eluate of the first chromatography contained a poly(A) fragment hybridizable to poly(U)-sepharose after RNAse action. It represents 15 % of messenger absorbance at 260 nm, which corresponds to an average length of 130 nucleotides, since the mean size of mRNAs is 900 nucleotides (to be published).

The same length for poly(A) was also obtained by polyacrylamide gel electrophoresis (fig. 1) conducted in formamide[6] to avoid error in molecular weight estimation[9].

These results suggest that half of the messenger activity is devoid of long poly(A) sequence.

2) Secondary structure of mRNA and quantification of its Poly(A) by a fluorescence method.

The method* is based on the ability of ethidium bromide to specifically intercalate in the double-stranded regions of

* This method was first applied to duck and rabbit globin mRNA[19]. It was presented by A. Favre at the workshop on mRNA's in eukaryotic cells held in Arolla (11-16 sept. 1972).

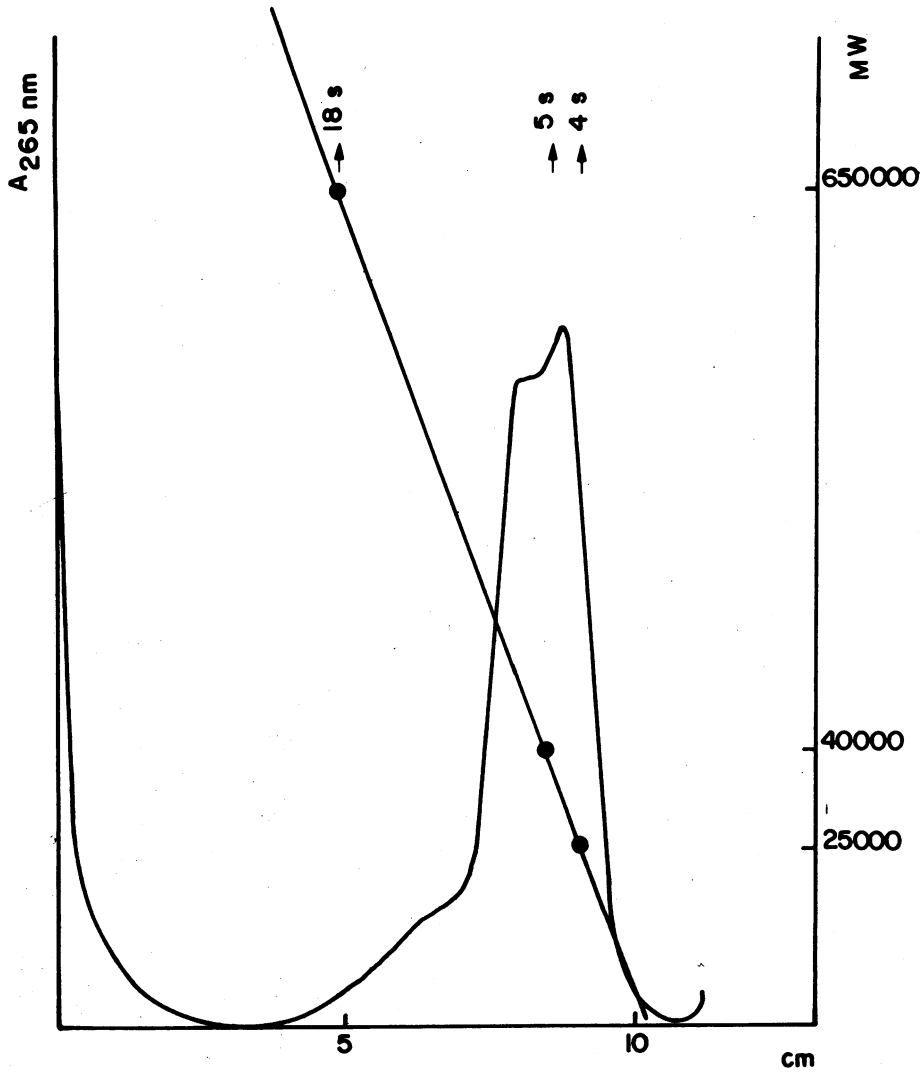

Fig. 1 - Polyacrylamide gel electrophoresis in formamide of poly(A) fragment. Poly(A) containing mRNA was isolated by poly(U)-sepharose chromatography from 7-17 S RNA fraction of zonal centrifugation. 15 A_{260} of this mRNA were hydrolysed by pancreatic and T_1 RNAse. The hydrolysate was precipitated with ethanol and chromatographed on poly(U)-sepharose. The eluate of the column was submitted to 2.5 % polyacrylamide gel electrophoresis in formamide for 4 h at 5 mA/gel.

nucleic acids, in a medium of moderate ionic strenght provide the concentration of the reagents remains low (see a review article on ethidium bromide properties in ref. 7). As shown by LE PECQ and PAOLETTI[18], the intercalation process is accompagnied by a considerable stimulation of the dye fluorescence. This technique has already been applied to the study of some RNA-protein interactions[26].

It is shown in table II that addition of mRNA stimulated the dye fluorescence. Moreover the stimulation was abolished by T_1 RNAse digestion. This reveals the existence of some secondary structure in our mRNA.

Table II

Evidence for secondary structure of mRNA

dye + mRNA	dye + digested mRNA	dye alone
10.6	5.8	4.7

Fluorescence - in arbitrary units - of ethidium bromide in the presence of intact and T_1 RNAse digested mRNA. These datas were deduced from figure 2 and represent the fluorescence signal in the absence of Poly(U).

A more quantitative estimation of the bihelical regions content can be deduced from the fluorimetric titration of mRNA by ethidium bromide (data not shown). The degree of base pairing, 45 to 60 %, is similar to that found by this method for duck and rabbit globin mRNA[19] and for calf lens mRNA[20].

The experimental principle used to detect the Poly(A) of our mRNA is as follows : the addition of synthetic Poly(U) will result in the formation of a new bihelical region that can be detected and quantified by mean of the ethidium bromide fluorescence. As shown in figure 2 the amount of poly(U) needed to saturate synthetic poly(A) was equal to the amount of poly(A) initially present. This method has been applied to the detection of poly(A) tract in globin mRNA[19] and calf lens mRNA[20].

Fluorescence was then measured in total 7-17 S RNA

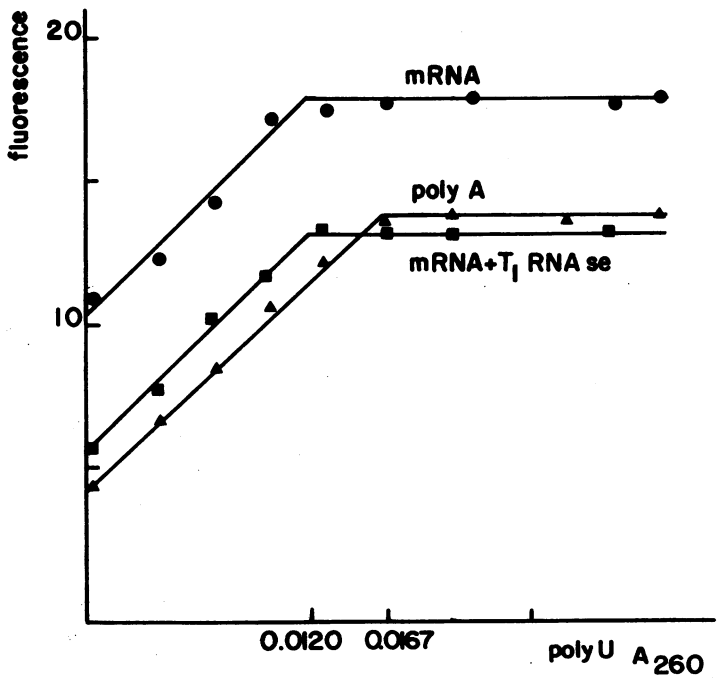

Fig. 2 - Detection by the ethidium bromide method of the poly(A) track of mammary mRNA purified by poly(U)-sepharose chromatography. Three solutions containing respectively 0.0615 A_{260} units of mRNA -●-●-●- , the same amount of mRNA digested by incubation for 1 h with 10 µl of 1 mg/ml T_1 RNAse solution -o-o-o-, 0.0167 A_{260} of synthetic poly(A) -□-□-□- were prepared in 2.10^{-6}M ethidium bromide, 0.05 M NaCl, 0.05 M sodium cacodylate buffer pH 7. The titration of each sample was performed at 25°C by stepwise addition of 3 µl aliquots of a 5.10^{-5}M poly(U) solution and the fluorescence signal (exc 540 nm - em 600 nm) read 2 min. after each addition.

fraction, in effluent and in eluate of 7-17 S RNA poly(U)-sepharose column. To the same amount of each RNA fraction was added an excess of poly(U) and ethidium bromide.

Results of table III, expressed in arbitrary units, show that the poly(A) content was low for 7-17 S total RNA, nil for effluent and very high for eluate. This fact is compatible with the lack of poly(A) sequence in mRNA not retained by poly(U)-sepharose.

Table III

FLUORESCENCE OF THE mRNA-POLY(U)-ETHIDIUM BROMIDE COMPLEX IN THE PRESENCE OF AN EXCESS OF POLY(U) AND ETHIDIUM BROMIDE
Experimental conditions are those of figure 2

	- poly(U)	+ poly(U)
Total 7-17 S RNA 0.05 A_{260}	115	122
Poly(U)-sepharose effluent 7-17 S RNA 0.05 A_{260}	119	120
Poly(U)-sepharose eluate 7-17 S RNA 0.05 A_{260}	96	209

Since the fluorescence method allows a quantitative determination of synthetic poly(A) (fig. 2) it was applied to the determination of the average length of poly(A) in our mRNAs. The slopes of the titration curves are identical for the intact mRNA, the digested mRNA and synthetic poly(A). This indicates that the poly(A) fragment of our samples is not interspersed with other residues as found with globin mRNA[19]. On the contrary in calf lens mRNA other residues seem to be present in the poly(A) segment[20]. The percentage of poly(A) in mRNA is expressed by :

$$\% \text{ poly(A)} = \frac{\epsilon_m}{\epsilon_A} \times \frac{A_{260} \text{ poly(A)}}{A_{260} \text{ mRNA}}$$

where

- ε_m is the mean molar extinction coefficient for a nucleotide included in RNA,

- ε_A is the molar extinction coefficient for A included in RNA,

- A_{260} poly(A) is the absorbance corresponding to poly(A) in mRNA. It was equal to A_{260} poly(U) added to reach the plateau,

- A_{260} mRNA was the absorbance of mRNA in the sample.

$$\% \text{ poly(A)} = \frac{8\,000}{10\,000} \times \frac{0.0120}{0.0615} \times 100 = 15\ \%$$

This value is in full agreement with that found by other experiments and it corresponds to an average poly(A) length of 130 nucleotides.

3) Nucleotide composition of poly(A) containing-mRNA

The base composition of poly(A) containing-mRNA revealed a very high proportion of AMP compared to that of rRNA (table IV). The comparison of mRNA and DNA composition indicated also an excess of AMP in mRNA. This excess can be evaluated to about 15 % if mRNA is assumed to reflect the composition of DNA.

This result is in full accordance with the poly(A) size determined by RNAse hydrolysis and polyacrylamide gel electrophoresis.

Table IV

NUCLEOTIDE COMPOSITION OF POLY(A) CONTAINING mRNA

	G	U	C	A	T
mRNA	16 %	23 %	22 %	40 %	
rRNA	29 %	32 %	32 %	19 %	
DNA	22 %		22 %	28 %	28 %

CONCLUSION

Poly(U)-sepharose chromatography of polysomal RNA led to the isolation of a mRNA fraction devoid of rRNA and tRNA, which corresponded to 0.8 - 1 % of the original polysomal RNA.

This mRNA fraction contains poly(A) sequence of 130 nucleotides average length estimated by several independent experiments. This is compatible with previous results on other mRNAs in which poly(A) is generally estimated to be 50-200 nucleotides long[10, 11, 12].

However, the poly(U)-sepharose column allows isolation of about half of the messenger molecules. This fact could be explained by the lack of poly(A) sequence in half of the mRNAs. In that case poly(A) sequence appears not necessary for faithfull and efficient translation. Comparison between messenger activity per A_{260} unit of mRNA with and without poly(A) could not be done since the mRNA fraction not retained by poly(U)-sepharose was never pure, even after zonal centrifugation. The 7-17 S mRNA fraction was completely devoid of 18 S rRNA but always heavily contaminated by other unknown RNAs (to be published). If messenger activity per A_{260} unit is similar for both kinds of mRNA, the total mRNA content of polysome must be 1.6 - 2 % of polysomal RNA.

The absence of poly(A) in many messengers molecules is not easy to explain. It seems not to be specific of α_s casein mRNA, since similar proportion was obtained for total messenger activity by TCA precipitation of total protein synthesized in a mRNA-dependent cell free system, and also for β and K casein messenger activity detected in reticulocyte lysate. It might be related to the very long half-life of membrane bound polysomes determined by incorporation of ^{3}H-uridine (unpublished results) since it has been observed that poly(A) segment of Hela cell mRNAs is becoming shorter with age[21]. Such a lack of poly(A) in some mRNAs has been already observed in Hela cells for histone[14] in chick oviduct for ovalbumin[15], in mouse reticulocytes for globins[13] and in yeast[10]. The existence in these mRNAs of oligo (A) too short to form a stable hybrid with poly(U) might easily explain these results. The poly(A) sequence might also contain other nucleotides in a proportion too high to

allow isolation of mRNA by the column. Although phenol extraction was demonstrated not to be responsible for poly(A) absence, an enzymatic[16] or chemical[17] cleavage of some poly(A) from mRNA cannot be completely excluded.

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