The polyA regions of hen oviduct RNA

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ABSTRACT

Total RNA from hen oviduct has been hydrolysed with a mixture of T_1 and pancreatic ribonucleases. Poly(A) tracts in the digestion product have been isolated by binding to oligo(dT) cellulose. Of the four major ribonucleotides, the product has been shown to contain only adenylic acid. When separated on polyacrylamide gels, the poly(A) gave two peaks corresponding to average apparent lengths of 270-280 and 540-550 nucleotides.

INTRODUCTION

The discovery several years ago that poly purines rich in adenylic acid were present in rat and mouse liver RNA [1, 2] and in globin mRNA [3] has led to many important advances in the study of mRNA. In particular, it has greatly facilitated the purification of messengers from various sources by the use of oligo(dT) cellulose [e.g. 4, 5, 6] and allowed the synthesis of DNA complementary to mRNA using reverse transcriptase from oncornaviruses [6, 7, 8].

However, no function is known for the poly(A) region, although several suggestions have been made. In particular, the observations of Brawerman [9] and of Sheiness and Darnell [10] lead to the conclusion that the poly(A) of newly synthesized mRNA is longer than that of older molecules and this in turn gives rise to the suggestion that the poly(A) region may in some way be involved in determining the lifetime of mRNA.

All previous experiments however have studied poly(A) by radioactive labelling techniques, principally by in vivo labelling. It seems possible that, if the poly(A) of different messengers is synthesized and processed at different rates, then these procedures could be observing only a portion of the total population. In any case, such studies cannot as yet give information which could relate poly(A) metabolism with the half-lives of individual messengers, which vary considerably [11, 12, 13, 14]. Such studies will be essential to test any hypothesis relating poly(A) length with the lifetime of mRNA.

We are attempting to overcome these difficulties by studying the mRNA of the hen oviduct. The purification and half-life of individual messengers has been well studied in this tissue [5, 13] and protein synthesis can be induced with hormones so that the maximum age of certain messengers, at intervals following induction, is known [15, 16, 17]. This paper reports our preliminary results. A procedure is described for improving the yield of total oviduct RNA for the isolation of mRNA, and the poly(A) in this RNA has been isolated and characterized by techniques not involving radioactive labelling.

MATERIALS & METHODS

Materials

AnalaR reagents were used when obtainable; RNase T₁ was obtained from Miles Research, Stoke Poges, Bucks. or Sankyo Co., Tokyo, Japan; pancreatic RNase A from Miles Research; Heparin, diethyl pyrocarbonate and 2', 3' phosphates of adenosine, cytidine, uridine and guanosine were from Sigma Chemical Co., London; Oligo(dT) cellulose from Searle Products for Research, High Wycombe, Bucks.; and HEPES from Calbiochem, San Diego, California.

Isolation of total RNA

The procedure was adapted from the method of Parish &

Kirby [18]. The magnum region of the oviduct of six laying hens, (White Leghorn), about 200g, was cooled and homogenized in 50 gm batches in an Atomix blender (MSE, Crawley, Sussex) for 1-2 mins. The homogenizing medium (total volume 1L) was a freshly prepared solution containing 0.5% sodium naphthalene disulphonate, 50 ug/ml heparin, 1% sodium 4-amino-salicylate. Just prior to use, 10 ml of 90% aqueous phenol containing 0.1% 8-hydroxy quinoline was added to the homogenizing medium. After homogenizing, one litre 12% 4-amino-salicylate at 4° was added and the mixture shaken vigorously by hand for 3 mins before the addition of one litre 90% phenol containing 0.1% 8-hydroxy quinoline, and a further 3 min shaking. One litre of chloroform was then added and, following a further 5 min shaking, the phases were separated by centrifuging at 2500g for 30 min at 10°.

The supernatant was decanted, solid NaCl added to 3% w/v and then a half-volume of a 1:1 mixture of 90% phenol (containing 0.1% 8-hydroxy quinoline) and chloroform and the mixture shaken for 10 mins at room temperature then centrifuged as above.

Two volumes of ethanol were added to the supernatant, and the bulk of DNA spooled out to avoid centrifuging it. The solution was then allowed to precipitate for 1 hr at -20° and the precipitate collected by centrifuging. The DNA and the RNA precipitate were washed with 75% ethanol, 2% sodium acetate pH6.0 at -20°, then dissolved in about 500 ml 1% sodium acetate, 100 ug/ml heparin pH6.0 at 4°. Undissolved material was removed by centrifuging (30,000g for 10 min) then RNA was precipitated by the addition of sodium chloride to 3M for 16 hr at 4°. The precipitate was collected by centrifuging (30,000g for 10 min) then washed four times with 200 ml 3M sodium acetate, twice with 75% ethanol, 2% sodium acetate, and once with ethanol.

The RNA was dried in vacuo or under a stream of nitrogen, dissolved in 10mM HEPES pH7.5 and stored at -20° .

This method permits the preparation of RNA from much larger quantities of tissue than can be handled by methods involving the preparation of polysomes [19]. In addition, the yield of RNA is doubled (about 420-450 mg per 100g tissue compared with 210-250 mg), without altering the specific activity of the product for the synthesis of ovalbumin. Analysis by polyacrylamide gel electrophoresis gives a profile essentially the same as that of RNA extracted from polysomes.

Enzymic hydrolysis of RNA and isolation of poly(A)

The procedure was that of Edmonds and Caramela [20] with minor modifications. RNA, 2 mg/ml, was hydrolysed with T_1 RNase, 400 units/ml, and pancreatic RNase 0.08 ug/ml in 0.01M HEPES pH7.5 for 15 min at 37° . The reaction was chilled in ice then shaken with an equal volume of phenol/chloroform (1:1v). The aqueous phase was made 3% with NaCl, then precipitated overnight with 2 volumes ethanol at -20° . The precipitate was washed with 75% ethanol 2% sodium acetate, then ethanol, and dried in vacuo. The product was dissolved in 50 ml 0.01M HEPES pH7.5, made 0.5M with KCl then shaken with 1g oligo(dT) cellulose for 15 min at room temperature. The cellulose was washed four times with 0.5M KCl by centrifuging briefly, then eluted four times by shaking with 0.01M HEPES pH7.5. The pooled eluates, total volume 50 ml, were made 0.1M with NaCl and precipitated with ethanol and the precipitate was collected and washed as above.

Polyacrylamide gel electrophoresis

Poly(A) samples ($1.5 A_{260}$ units) were separated on 2.4% and 5% polyacrylamide gels as described by Loening [21], or denaturing polyacrylamide gels as described by Haines et al [5] except that the buffer for the preparation of gels and the running buffer contained 36 mM tris 20 mM NaH_2PO_4 1mM EDTA and

electrophoresis was carried out at room temperature.

Hydrolysis of poly(A)

A sample of the material eluted from oligo(dT) cellulose was mixed with an equal volume of 0.6M KOH and hydrolysed for 16 h at 37°. The hydrolysate was acidified with 3M HClO₄ and the precipitate removed by centrifuging. The supernatant was then neutralized with KOH using phenol red as indicator and again centrifuged.

Thin layer chromatography

Samples of the hydrolysate (0.1 A₂₆₀ units) were chromatographed on PEI-cellulose thin layer plates (E. Merck, A.G. Darmstadt) as described by Randerath and Randerath [22]. Samples (2 ul of 0.01M solutions) of the 2', 3' mononucleotides were used as markers.

RESULTS AND DISCUSSION

In a typical experiment, 26,650 A₂₆₀ units of total RNA which would normally be obtained from the oviducts of about 25-30 chickens, yielded 43 A₂₆₀ units of material presumed to be poly(A). A sample of the supposed poly(A) was hydrolysed with KOH, as described in the methods section, and about 0.1 A₂₆₀ units separated by thin layer chromatography (Figure 1). In each case only one nucleotide, which coincided with the adenylic acid marker, was found. The thin layer procedure separated the 2' and 3' phosphates of adenylic acid, but not of the other nucleotides. The presence of both forms in the products of hydrolysis of the enzyme digested mRNA gives further confidence that the polymer contained adenylic acid, and no detectable quantity of the other common ribonucleotides. This result has been confirmed by subjecting the KOH hydrolysate to high voltage paper electrophoresis according to Barrell [23]. A single spot was observed which coincided with the adenylic acid marker.

Samples of the poly(A) from hen oviduct RNA were subjected to gel electrophoresis as described in the Methods section. UV profiles of the gels are shown in Figure 2a curve (i). The product separated mainly into two rather broad peaks with apparent molecular weights of about 90,000 and 180,000 at the peaks when compared with 4s 18s and 28s RNA markers (Figure 3). These values, equivalent to 275 and 550 adenine residues per chain, must be taken as upper limits since it has been suggested that poly(A) is anomalously retarded on gels [24, 25]. Comparison with authentic poly(A) markers has not yet been possible, however, using the data of Pinder and Gratzner [26] estimates of 70,000 and 150,000 daltons are obtained, equivalent to 210 and 450 nucleotides at the peaks. These values are much larger than has been shown for the poly(A) of other tissues and organisms [e.g. 10, 25, 27].

There are several ways in which the existence of a double peak in the poly(A) of our preparations could be an artefact of preparation and not present in the cellular messengers. We have examined some of these possibilities.

It has been reported that under low salt conditions, T_1 ribonuclease can hydrolyse RNA adjacent to adenylic acid residues, rather than guanylic acid [28]. The possibility that the double peak found in oviduct poly(A) resulted from such anomalous splits was checked by carrying out the nuclease treatment of the total RNA in 0.15M NaCl. As shown in Figure 2a, curve (ii), this treatment did not alter the distribution of material in the poly(A) recovered.

A second suggestion to account for the bimodal distribution of the poly(A) is that the peak of higher molecular weight is a dimer of the lower molecular weight material. This was tested by running the gels under denaturing conditions (Figure 2b) [5]. The distribution of material was unchanged, showing that both peaks are due to mixtures of individual molecular species, and

Figure 1 Thin layer chromatogram of hydrolysed poly(A) (tracks X and Y) and 2' 3' nucleoside phosphate markers (A = adenosine, G+C = guanosine plus cytosine, U = uridine).

Figure 2 a Electrophoresis of hen oviduct poly(A) on a 5% polyacrylamide gel. Curve (i), total RNA treated with nuclease under low salt conditions. Curve (ii), total RNA treated with nuclease under high salt conditions.

b Electrophoresis of hen oviduct poly(A) on a 2.4% polyacrylamide gel in formamide.

The heavy arrows give the position of 4S marker on separate gels and the light arrow of the tracker dye on the formamide gel.

Figure 3 Electrophoresis of 1.5 A260 units hen oviduct poly(A) in the presence of 1.5 A260 units each of oviduct polysomal RNA and rat liver 4S RNA on a 2.4% polyacrylamide gel. The heavy arrows give the position of the 28S and 18S RNA, and the light arrow of the 4S RNA.

not to aggregates.

A third possibility is that our method of preparation extracts nuclear RNA in combination with cytoplasmic RNA and that it is nuclear messenger which provides the poly(A) of one of the two peaks. In view of the very small amounts of mRNA found in the nucleus compared with cytoplasm (less than 2%, S. Emtage, personal communication), it is not possible that the presence of nuclear RNA could account for the amount of material in either of the two major poly(A) peaks. Nuclear RNA however is known to contain smaller oligomers of adenylic acid [29] so that this could be the source of some of the low molecular weight material seen on the gels in figure 2.

The poly(A) tracts of eukaryotic cells and viruses observed by in vivo labelling techniques have not shown a bimodal distribution [e.g. 10, 30, 31] although some viral RNAs contain poly(A) tracts of two size classes [32, 33]. It was found recently by Mansbridge et al [34] that mixed globin α and β chain mRNA of reticulocytes labelled enzymically and chemically after the isolation of the messengers contain two broad poly(A) peaks. It is possible that these differences are due to the method of observation and not to inherent differences in the poly(A) tracts of the different RNA molecules.

It would therefore appear that the double peak of poly(A) is not an artefact and we may now discuss how this could arise in the usual lifetime of messengers. There are at least three possibilities. The first possibility arises from the consequences of the shortening of poly(A) which occurs after messengers leave the nucleus. If the ticketing hypothesis of Sussman [35] as applied to poly(A) is correct, that is that the half-life of a messenger is determined by the shortening of the poly(A), and if all messenger species start functioning with poly(A) tracts of the same length, then the rate of shortening must differ for

each mRNA species to account for differences in messenger half-lives. Alternatively, if the rate of shortening of all poly(A) tracts is constant, then each messenger must start with a poly(A) tract whose length is related to its half-life. The situation could be between these extremes. The total poly(A) of a cell, observed by the methods we have chosen, will be a complex sum relating the maximum length of the poly(A) of each messenger, its rate of shortening and the relative abundance of each messenger. A bimodal distribution such as we have observed could therefore be the result of this summation. A second possibility, not precluded by the complexities discussed above, can be derived from the fact that in the hen oviduct, ovalbumin mRNA represents about half the total messenger population. Thus one of the two peaks observed in the total poly(A) could be derived entirely from the ovalbumin messenger. Finally, it is possible that each messenger in the population has poly(A) tracts covering both peaks observed in the total extract.

Clearly a choice between these alternatives will be of great importance in deciding whether the ticketing hypothesis provides a function for poly(A). At the moment, there is insufficient evidence to make this choice.

It is also worth noting that if the proportion of the four nucleotides in the remainder of the molecules is approximately equal, then the amount of adenylic acid added by the poly(A) to a molecule of the size of ovalbumin mRNA (875,000 daltons [5]) would give rise to a base composition significantly rich in adenylic acid, particularly if the largest size classes observed were present.

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REFERENCES

- 1 Hadjivassiliou, A. & Brawerman, G. (1966) J. Mol. Biol. 20 1-7.
- 2 Lim, L., Canellakis, Z.N. & Canellakis, E.S. (1970) Biochim. Biophys. Acta 209 128-138.
- 3 Lim, L. & Canellakis, E.S. (1970) Nature (London) 227 710-712.
- 4 Aviv, H. & Leder, P. (1972) Proc. Nat. Acad. Sci. USA 69 1408-1412.
- 5 Haines, M.E., Carey, N.H. & Palmiter, R.D. (1974) Eur. J. Biochem. 43 549-560.
- 6 Ross, J., Aviv, H., Scolnick, E. & Leder, P. (1972) Proc. Nat. Acad. Sci. USA 69 264-268.
- 7 Verma, I.M., Temple, G.F., Fan, H. & Baltimore, D. (1972) Nature New Biol. (London) 235 163-167.
- 8 Kacian, D.L., Spiegelman, S., Bank, A., Terada, M., Metafora, S., Dow, L. & Marks, P.A. (1972) Nature New Biol. (London) 235 167-169.
- 9 Brawerman, G. (1973) Molec. Biol. Rep. 1 7-13.
- 10 Sheiness, D. & Darnell, J.E. (1973) Nature New Biol. (London) 241 265-268.
- 11 Tilghman, S.M., Hanson, R.W., Reshef, L., Hopgood, M.F. & Ballard, F.J. (1974) Proc. Nat. Acad. Sci. USA 71 1304-1308.
- 12 Palmiter, R.D. & Schimke, R.T. (1973) J. Biol. Chem. 248 1502-1512.
- 13 Palmiter, R.D. (1973) J. Biol. Chem. 248 8260-8270.
- 14 Palmiter, R.D. & Carey, N.H. (1974) Proc. Nat. Acad. Sci. USA 71 2357-2361.
- 15 Chan, L., Means, A.R. & O'Malley, B.W. (1973) Proc. Nat. Acad. Sci. USA 70 1870-1874.
- 16 Palmiter, R.D. (1972) J. Biol. Chem. 247 6770-6780.
- 17 Rhoads, R.E., McKnight, G.S. & Schimke, R.T. (1973) J. Biol. Chem. 248 2031-2039.
- 18 Parish, J.H. & Kirby, K.S. (1967) Biochim. Biophys. Acta 142 273-275.
- 19 Palmiter, R.D. (1973) J. Biol. Chem. 248 2095-2106.
- 20 Edmonds, M. & Caramela, M.G. (1969) J. Biol. Chem. 244 1314-1324.
- 21 Loening, U.E. (1967) Biochem. J. 102 251-257.
- 22 Randerath, E. & Randerath, K. (1965) Anal. Biochem. 12 83-93.
- 23 Barrell, B.G. (1971) in Procedures in Nucleic Acids Research eds. Cantoni, G.L. & Davis, D.R. (Harper & Row New York) 751-779.
- 24 Fraser, R.S.S. & Loening, U.E. (1973) Eur. J. Biochem. 34 153-158.
- 25 Morrison, M.R., Merkel, C.G. & Lingrel, J.B. (1973) Mol. Biol. Reports 1 55-60.
- 26 Pinder, J.C. & Gratzner, W.B. (1974) Biochim. Biophys. Acta 349 47-52.

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- 27 Yogo, Y. & Wimmer, E. (1972) *Proc. Nat. Acad. Sci. USA* 69 1877-1882.
 - 28 Eiden, J.J. & Nichols, J.L. (1973) *Biochemistry* 12 3951-3956.
 - 29 Nakazato, H., Edmonds, M. & Kopp, D.W. (1974) *Proc. Nat. Acad. Sci. USA* 71 200-204.
 - 30 Edmonds, M., Vaughan, M.H. & Nakazato, H. (1971) *Proc. Nat. Acad. Sci. USA* 68 1336-1340.
 - 31 Pridgen, C. & Kingsbury, D.W. (1972) *Journal of Virology* 10 314-317.
 - 32 Banerjee, A.K. & Rhodes, D.P. (1973) *Proc. Nat. Acad. Sci. USA* 70 3566-3570.
 - 33 Eaton, B.T. & Faulkner, P. (1972) *Virology* 50 865-873.
 - 34 Mansbridge, J.N., Crossley, J.A., Lanyon, W.G. & Williamson, R. (1974) *Eur. J. Biochem.* 44 261-269.
 - 35 Sussman, M. (1970) *Nature (London)* 225 1245-1246.