A MODEL FOR THE ORGANIZATION OF THE POLY(A) · PROTEIN COMPLEX IN MESSENGER RIBONUCLEOPROTEIN

David S. ADAMS[+], Daniel NOONAN and William R. JEFFERY

[+]*Department of Cell Biology, The Rockefeller University, New York, NY 10021* and *Department of Zoology, University of Texas, Austin, TX 78712, USA*

Received 21 March 1980

1. Introduction

The messenger RNA of eukaryotic cells exists in the form of a messenger ribonucleoprotein particle (mRNP) [1,2]. A major site of protein interaction in this particle is the poly(A) sequence which forms a poly(A) · protein complex at the 3′-terminus of its mRNA moiety [3–6]. Although [4,6–16] have focused on describing the poly(A) and protein moieties of this complex and the possible roles they play in the cell [9,17] no information is available on the structural organization of the particle. The extent and position of protein interaction along the poly(A) sequence, for instance, is unknown. The organization of the poly(A) · protein complex is a problem of considerable importance in molecular biology since the poly(A) moiety of this structure has been proposed to govern the metabolic stability of mRNA [18–20]. Here we present a model for the poly(A) · protein complex and present evidence supporting its validity based on micrococcal nuclease digestion experiments.

2. Methods

The slime mold, *Physarum polycephalum* was used as a source of the poly(A) · protein complex. Plasmodia were cultured and labeled for 2 h with [^{3}H]adenosine (48 Ci/mmol; ICN Radiochemicals, Irvine, CA) at 20 μCi/ml as in [21]. Labeled plasmodia were collected, homogenized [21], and the homogenate was centrifuged for 10 min at 20 000 × *g* to obtain a post-mitochondrial supernatant. Poly(A) · protein complexes were detached from the supernatant mRNP by treatment with a mixture of pancreatic and T_1 RNases [22] and ribosomes were removed from the digest by differential centrifugation [14]. The supernatant of this centrifugation contains the poly(A) · protein complex.

Micrococcal nuclease digestion was employed to determine the extent of poly(A) sequence interaction with protein in the complexes. The sensitivity of poly(A) · protein complexes dissolved in 50 mM Tris–HCl (pH 7.6), 100 mM NaCl, and 1 mM $MgCl_2$ was tested by adding $CaCl_2$ and enzyme to final concentrations of 20 mM and 500 μg/ml, respectively, and incubating the mixture at 4°C for 40 h. Control aliquots of poly(A) · protein complex prepared in parallel were incubated under the same conditions except that micrococcal nuclease was not added. The incubations were carried out at 4°C because at 25°C the *Physarum* poly(A) · protein complex showed endogenous nucleolytic activity. These digestion conditions yield a poly(A) limit digest [22]. At the end of the incubation period the reaction was terminated by adding EGTA to final conc. 20 mM. The digests and controls were then deproteinized by treatment with 500 μg/ml proteinase K for 12 h at 4°C. The radioactivity in poly(A) was measured by passing the digest through poly(U)-impregnated glass fiber filters. The size of poly(A) sequences was determined by electrophoresis through 7 cm long, 6.5% acrylamide–0.23% bisacrylamide gels. Electrophoresis was carried out at 1 mA/gel until the bromphenol blue marker dye had traveled ~5 cm through the gel [21]. Following electrophoresis the gels were fractionated into 2 mm slices, the slices were eluted in a small volume of 50 mM Tris–HCl (pH 7.6), 100 mM NaCl, and 1 mM $MgCl_2$ for 24 h at 37°C, and the eluates were placed in 10 ml aquasol and counted.

Address correspondence to W.R.J.

3. Models

Some possible models for poly(A) · protein complex organization are presented in fig.1. Protein binding sites alternate along the poly(A) segment between protein-free regions in the first model (fig.1A). The poly(A) sequence is entirely covered by protein in the second model (fig.1B). In the last three models the protein binding site is associated with a large, contiguous sector of the poly(A) sequence but, some areas, either in the 5′-terminal region (fig.1D), the 3′-terminal region (fig.1E), or both (fig.1C), remain unoccupied. The purpose of this paper is to report experiments which distinguish between these models and determine the location of the protein binding site in the poly(A) sequence.

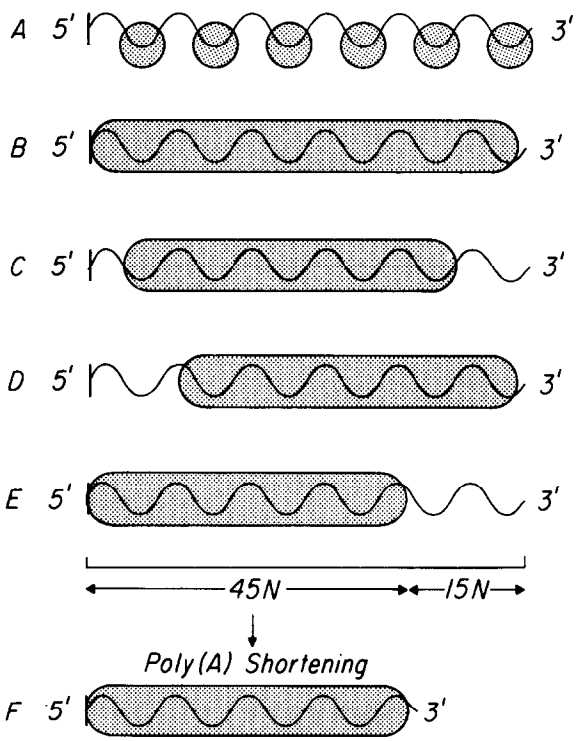

Fig.1. Models for the organization of the poly(A) · protein complex. The heavy wavy lines represent poly(A) sequence. The shaded areas represent poly(A) binding proteins or protein binding sites. The heavy vertical lines represent the border of poly(A) sequence and the adjacent mRNA sequence. The salient features of models A–E are discussed in the text. Model E as it would appear following cytoplasmic poly(A) shortening is represented in G. The 5′ and 3′ termini of the sequences are indicated.

4. Results and discussion

Micrococcal nuclease digestion was utilized to study the organization of the poly(A) · protein complex. This enzyme completely hydrolyzes deproteinized *Physarum* poly(A) [22] and is effective on single or double-stranded nucleic acid substrates that are not protected by protein associations [23]. After micrococcal nuclease digestion for 40 h at 4°C under standard assay conditions 70–80% (4 expt.) of the original adenosine radioactivity in the poly(A) · protein complex was retained by poly(U) filters after deproteinization. The incomplete resistance of the poly(A) sequence to digestion appears to exclude the second model for poly(A) · protein complex organization (fig.1B) in which the poly(A) tract is entirely protected by protein. Since the size of the protected fragments is not revealed in this experiment it does not discriminate between the other models.

In order to determine the size of the protected fragments the micrococcal nuclease digests were deproteinized and subjected to polyacrylamide gel electrophoresis. As shown in fig.2, the poly(A) fragments from the limit digest migrate more rapidly than intact poly(A) from untreated controls. In *Physarum* the number average size of poly(A) in the poly(A) · protein complexes labeled for 2 h is about 60 nucleotides (fig.2A). We estimate the number average size of the digested poly(A) to be 45 nucleotides (fig.2B). This represents a size reduction of 25% of the sequence and is compatible with 20–30% loss in total poly(A) radioactivity observed following micrococcal nuclease digestion.

Since relatively large poly(A) fragments remain after micrococcal nuclease digestion our first model, in which small protein-free and protein associated regions are interspersed along the poly(A) sequence (fig.1A), also appears to be untenable. Instead the data are consistent with structures like those depicted in the last three models (fig.1C–E). In these relatively large, contiguous regions of the poly(A) sequence are protected by protein. Further experiments were designed to determine the location of the protein binding site.

As mRNA ages in the cytoplasm its poly(A) sequence is gradually shortened [24–28]. In *Physarum* mRNA the shortening process involves the removal of 15–20 adenylate residues leaving a poly(A) sequence 45–50 nucleotides long at steady-state [21]. The proportion of poly(A) protected from micrococcal

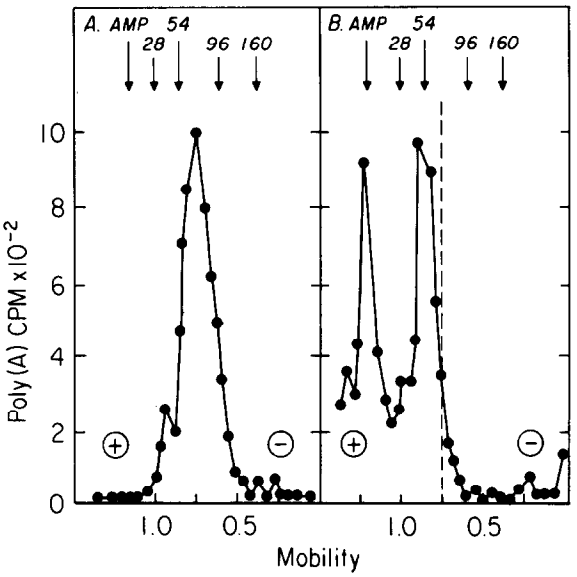

Fig.2. Polyacrylamide gel electrophoresis of poly(A) sequences in the limit digest of the micrococcal nuclease-digested poly(A) · protein complex. The electrophoretic mobility of poly(A) derived from the untreated poly(A) · protein complex is shown in A while that of poly(A) derived from the micrococcal nuclease limit digest is shown in B. The vertical dashed line in B represents the average mobility of poly(A) from the untreated complex in A. The vertical arrows represent the average mobilities of adenosine monophosphate and synthetic poly(A) of average lengths of 28, 64, 96, and 160 nucleotides which were electrophorezed on separate gels.

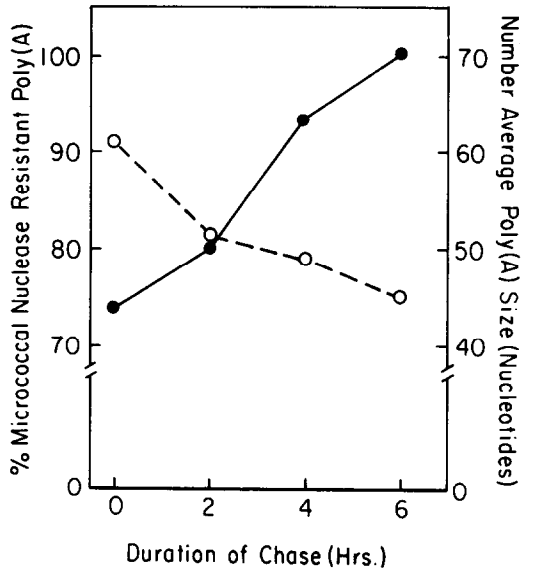

Fig.3. The relationship of poly(A) shortening to the proportion of micrococcal nuclease resistance exhibited by the poly(A) moiety of the poly(A) · protein complex during a pulse–chase labeling experiment. A culture was labeled with [^{3}H]adenosine for 2 h then chased with conditioned medium containing 1 mM adenosine as in [21]. Poly(A) · protein complexes were isolated from aliquots taken from the culture at various times during the chase, assayed for micrococcal nuclease resistance, deproteinized, and the amount of sequence resistance was determined by poly(U) filtration. The proportion of micrococcal nuclease-resistant poly(A) is indicated by the closed circles. The open circles represent the number average size of poly(A) sequences during mRNA aging calculated from the electrophoretic mobilities of poly(A) sequences isolated from cytoplasmic RNA during an equivalent pulse–chase experiment.

nuclease digestion after shortening should be dependent on the position of the protein binding site in the sequence. Localization of this site in a region including the 3′-terminus sector (fig.1B) would be expected to reduce the proportion of resistant sequence since shortening would remove nucleotides that normally interact with protein and are thus resistant to hydrolysis. Positioning of the protein-protected sector in the 5′-region (fig.1E), however, would increase the proportion of resistant sequence since shortening would affect only the unprotected nucleotides. If the protein-binding sectors were located internally (fig.1C) an intermediate result might be attained. In order to test these possibilities we labeled a culture with [^{3}H]-adenosine for 2 h and then initiated a chase to observe poly(A) shortening [21]. Aliquots were removed during the chase and the poly(A) · protein complexes derived from them were tested for micrococcal nuclease sensitivity. As shown in fig.3 the proportion of sequence resist to enzymatic hydrolysis gradually increased as poly(A) shortening progressed. Eventually 100% of the sequence became resistant to the enzyme. The results support the possibility that poly(A) sequences in *Physarum* mRNP contain a long protein binding site beginning at or near their 5′ ends.

The simplest interpretation of all our results is that a protein is located in the sector of poly(A) sequence of *Physarum* beginning at or near the 5′-end and extending for about 45 nucleotides toward the 3′-end (fig.1E). This leaves the last 15 or so adenylate residues free of protein and presumably available for shortening by cytoplasmic enzymes (fig.1E,F). This model also explains the ability of the poly(A) · protein complex to form base-paired hybrids with poly(U) [3,5] and oligo (dT)-cellulose [29] and the enhancement of poly(U) hybridization following deproteiniza-

tion [30]. Assuming poly(A) exists as a single-stranded, helical rod with a pitch of 3.4 Å at cellular pH [31], a 45 nucleotide tract completely covered by protein would require about 153 Å of associated polypeptide sequence. A binding site of this size could be accomodated by one copy of the 78 000 mol. wt poly(A) binding protein [4], as stoichiometric calculations suggest [32], if this polypeptide were only about 10–20% α-helix.

It might be questioned as to why the poly(A) binding protein could specifically bind to the 5′-region of a presumably homogenous polynucleotide sequence as implied by our model. This could be readily explained if the poly(A) binding protein also shows affinity for binding sites in the mRNA sequence adjacent to the poly(A) segment. This organization feature in the poly(A) · protein complex, which is supported by some experimental evidence [6,33], could be involved in the regulation of mRNA stability in eukaryotic cells.

Acknowledgements

This work was supported by grants PCM-77-24767 from the National Science Foundation and GM-24119 from the National Institutes of Health.

References

[1] Greenberg, J. (1975) J. Cell. Biol. 64, 269–288.
[2] Preobrazhensky, A. and Spirin, A. (1978) Prog. Nucleic Acid Res. Mol. Biol. 21, 1–27.
[3] Kwan, S.-W. and Brawerman, G. (1972) Proc. Natl. Acad. Sci. USA 69, 3247–3250.
[4] Blobel, G. (1973) Proc. Natl. Acad. Sci. USA 70, 924–928.
[5] Jeffery, W. R. and Brawerman, G. (1974) Biochemistry 13, 4633–4637.
[6] Jeffery, W. R. (1978) Biochim. Biophys. Acta 521, 217–228.
[7] Kumar, A. and Pederson, T. (1975) J. Mol. Biol. 96, 353–365.
[8] Kish, V. M. and Pederson, T. (1976) J. Biol. Chem. 251, 5888–5894.
[9] Irwin, D., Kumar, A. and Malt, R. (1975) Cell 4, 157–165.
[10] Firtel, R. A. and Pederson, T. (1975) Proc. Natl. Acad. Sci. USA 72, 301–305.
[11] Barrieux, A., Ingraham, H., Nystul, S. and Rosenfeld, M. (1976) Biochemistry 15, 3523–3528.
[12] Schwartz, H. and Darnell, J. E. (1976) J. Mol. Biol. 104, 833–851.
[13] Van Venrooij, W., von Eekelen, C., Jansen, R. and Princzen, J. (1977) Nature 270, 189–191.
[14] Jeffery, W. R. (1977) J. Biol. Chem. 252, 3525–3532.
[15] Cardelli, J. and Pitot, H. (1977) Biochemistry 16, 5127–5134.
[16] Peters, C. and Jeffery, W. R. (1978) Differentiation 12, 91–97.
[17] Rose, K., Jacob, S. and Kumar, A. (1979) Nature 279, 260–262.
[18] Hieter, P., LeGendre, S. and Levy, C. (1976) J. Biol. Chem. 251, 3287–3293.
[19] Marbaix, G., Huez, G. and Soreq, H. (1977) Trends Biochem. Sci. 2, N106–N107.
[20] Sheiness, D., Puckett, L. and Darnell, J. E. (1975) Proc. Natl. Acad. Sci. USA 72, 1077–1081.
[21] Adams, D. S. and Jeffery, W. R. (1978) Biochemistry 17, 4519–4524.
[22] Adams, D. S., Noonan, D. and Jeffery, W. R. (1980) Biochemistry, in press.
[23] Alexander, M., Heppel, L. and Hurwitz, J. (1961) J. Biol. Chem. 236, 3014–3019.
[24] Sheiness, D. and Darnell, J. E. (1973) Nature New Biol. 241, 265–268.
[25] Brawerman, G. (1973) Mol. Biol. Rep. 1, 7–13.
[26] Merkel, C. G., Wood, G. T. and Lingrel, J. B. (1976) J. Biol. Chem. 251, 5512–5515.
[27] Jaworski, A., Wu, W. and Bateman, O. (1976) Arch. Biochem. Biophys. 177, 516–524.
[28] Wilson, M. C., Sawicki, S. G., White, P. A. and Darnell, J. E. (1978) J. Mol. Biol. 126, 23–36.
[29] Lindberg, U. and Sundquist, B. (1974) J. Mol. Biol. 86, 451–468.
[30] Favre, A., Morel, C. and Scherrer, K. (1975) Eur. J. Biochem. 57, 147–157.
[31] Brahms, J., Michelson, A. M. and Van Holde, K. E. (1966) J. Mol. Biol. 15, 467–488.
[32] Kish, V. M. and Pederson, T. (1975) J. Mol. Biol. 95, 227–238.
[33] Bergmann, I. and Brawerman, G. (1977) Biochemistry 16, 259–264.