A Micro-Method for the Assay of Polyadenylate-Containing Ribonucleic Acid by Gel Electrophoresis

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(Received 4 June 1975)

A micro-method for the determination of the electrophoretic profile of the various poly(A)-containing RNA species in a RNA sample was developed. The method is simple to carry out and allows for great reproducibility. It combines the resolving power of electrophoresis in agarose with the specificity of binding of poly(A) to poly(U)-containing glass-fibre filters. It consists of the following steps. (1) The molecules in an RNA sample are first separated according to their molecular weight by electrophoresis in agarose, at low ionic strength. 2. The molecules thus separated are then submitted to a second electrophoresis in 'binding buffer' in a direction perpendicular to the first one. In the course of this electrophoresis the poly(A)-containing RNA species are separated from other RNA species as they bind to a poly(U)-containing glass-fibre filter which is placed across the electrophoresis path. The method was used to determine the electrophoretic profile of *Rhynchosciara* salivary-gland messenger RNA. It was found that the population of messenger RNA in the gland is dominated by forms moving as 18 and 15S peaks, but there is also polydisperse RNA with slower mobility.

The fact that most eukaryotic mRNA molecules contain sequences of poly(A) attached to their 3'-end (Edmonds & Caramela, 1969; Lim & Canellakis, 1970; Darnell *et al.*, 1971; Lee *et al.*, 1971; Mendecki *et al.*, 1972; Molloy *et al.*, 1972; Molloy & Darnell, 1973) has proved to be of great use in the study of mRNA. Several methods based on this fact have been developed which make it possible to detect poly(A)-containing sequences. These methods depend on the binding of mRNA to Millipore filters (Lee *et al.*, 1971) or to cellulose alone at high ionic strength (Schutz *et al.*, 1972; Sullivan & Roberts, 1973; Kitos *et al.*, 1974), binding to poly(U) or poly(dT) fixed to cellulose (Edmonds & Caramela, 1969; Kates, 1970; Aviv & Leder, 1972; Sheldon *et al.*, 1972; Morrison *et al.*, 1972) or to poly(U) fixed to Sepharose (Adesnik *et al.*, 1972; Jelinek *et al.*, 1973) or glass-fibre filters (Sheldon *et al.*, 1972).

To obtain information about the different mRNA species in an RNA sample, it is possible to fractionate the RNA in a sucrose gradient and then assay for the poly(A) content of each fraction (Lee *et al.*, 1971) or challenge each sample with radioactive poly(U) (Bishop *et al.*, 1974). Fraser & Loening (1973) also developed a method in which the sample to be analysed is previously hybridized with radioactive poly(U) and then fractionated by sucrose-gradient centrifugation or gel electrophoresis. The different poly(A)-containing molecules are then characterized by their mobility under either fractionation procedure.

We have now developed a micro-method for the fractionation of poly(A)-containing molecules which combines the simplicity of binding to poly(U) fixed to a glass-fibre filter with the high resolution of gel electrophoresis in agarose. In the present paper we describe the experiments which led to the establishment of this method, and the results obtained by its application to determine the electrophoretic profile of *Rhynchosciara* salivary-gland messenger RNA.

Materials and Methods

Animals

Larvae from the fly *Rhynchosciara americana* were cultured under laboratory conditions as described by Lara *et al.* (1965). We used animals in late fourth instar (approx. age 46-49 days), at the time at which the larvae start to spin their communal cocoon.

RNA labelling and extraction

For this purpose we used the methods developed in Edstrom's laboratory (Daneholt, 1972) with some modifications. The salivary glands were obtained by dissection, and incubated for 3 h at 20°C in a physiological solution for *Rhynchosciara* (Terra *et al.*, 1975) which contained 100 μ Ci of [^{3} H]uridine (29 Ci/mmol; NEN). They were then homogenized in 0.02M-Tris-HCl, pH7.4, containing 0.5% sodium

dodecyl sulphate; Pronase (B grade; Calbiochem, Los Angeles, Calif., U.S.A.) was added to a final concentration of 1 mg/ml and digestion carried out at 25°C for 10 min. In our case, higher temperatures led to degradation, particularly of mature rRNA (M. C. Okretic & F. H. S. Lara, unpublished work). The RNA was precipitated from the digest by addition of 2.5 vol. of 70% (v/v) ethanol in 0.2 M-potassium acetate (Lambert *et al.*, 1972).

Oligo(dT)-cellulose chromatography

The procedure of Aviv & Leder (1972) was used. The RNA sample to be analysed was suspended in high-ionic-strength buffer (0.5 M-KCl, 0.01 M-Tris-HCl, pH 7.4) and applied to a column of oligo(dT)-cellulose equilibrated with the same buffer. The column was washed with 8 ml of the high-ionic-strength buffer in order to eliminate the non-adsorbed RNA. The bound RNA was then eluted from the column with 8 ml of the low-ionic-strength buffer (0.02 M-Tris-HCl, pH 7.4) and precipitated by the addition of 2.5 vol. of 70% ethanol in 0.2 M-potassium acetate (Lambert *et al.*, 1972). All operations were carried out at 25°C and all buffers used were pretreated with diethyl pyrocarbonate (Solymosy *et al.*, 1968). The oligo(dT)-cellulose (synthesized by the method of Aviv & Leder, 1972) was a kind gift from Dr. H. Westphal.

Two-dimensional electrophoresis for the separation of poly(A)-containing RNA

A diagrammatic representation of the method is shown in Fig. 1. A 50 µl sample of RNA was placed in a 29 mm × 3 mm × 1 mm trough in a 4 cm × 9 cm × 0.3 mm slab of 2% agarose in 0.02 M-Tris-HCl, pH 8, 0.02 M-NaCl, 0.002 M-EDTA (E buffer) with 0.2% sodium dodecyl sulphate as described by Daneholt *et al.* (1969). Electrophoresis was then conducted for 100 min at 10 mA/cm² in a chamber described by Wieme (1965). At the end of this electrophoresis two equal gel lanes were cut from the gel slab in the direction of the RNA migration path. One of these was scanned in the u.v. in order to determine the position of the rRNA and tRNA bands and, after this, it was cut into 2 mm fractions and assayed for radioactivity to determine the position of the labelled species. The other gel slab was then welded along its longer dimension, with the help of 1% agarose in 0.01 M-Tris-HCl, pH 7.4, 0.12 M-NaCl, 0.5% sodium dodecyl sulphate (H-E buffer) to the longest dimension of a 9 mm × 4 mm × 0.3 mm slab of 1% agarose in H-E buffer. This slab contained, at 5 mm from its 9 cm edge, a glass-fibre filter to which poly(U) had been attached by the procedure of Sheldon *et al.* (1972). Electrophoresis was then carried out for 6 h at 25°C in a direction perpendicular to the direction of the first electrophoresis (see Fig. 1).

In this way, the molecules separated in the first electrophoresis migrated in parallel during this second electrophoresis and moved through the poly(U)-containing glass-fibre filter. The poly(A)-containing molecules bound to the filter as they reached it. At the end of the run the glass-fibre filter was removed and cut into fractions (2 mm) which were assayed for radioactivity.

Radioactivity assay

The samples (either agarose, or glass-fibre-filter sections) were placed in plastic mini-vials and covered with 2.7 ml of a solution of 5 mg of 2,5-diphenyloxazole and 0.5 mg of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene containing 30 ml of Protosol (New England Nuclear, Boston, Mass., U.S.A.) solubilizer. After standing overnight, the mini-vials were placed inside normal scintillation vials and counted for radioactivity on a Beckman LS-250 liquid-scintillation spectrometer with an efficiency of 43% for ³H.

Results

Preliminary experiments

The design of the two-dimensional electrophoresis, which is detailed in the Materials and Methods section, would only be feasible if the RNA moved sufficiently on electrophoresis at the high ionic strength, that is essential for the hybridization of poly(A) to poly(U). Thus we electrophoresed separately, in 1% agarose in H-E buffer, both synthetic poly(A) and a sample of *Rhynchosciara* salivary-gland ³H-labelled RNA which we found to contain radioactivity as poly(A)-containing RNA by the procedure of Sheldon *et al.* (1972). We found that under these conditions both samples moved to considerable distances, as indicated in the profiles shown in Fig. 2.

It was also necessary to determine whether there was any non-specific binding of RNA to the poly(U)-containing glass-fibre filter on electrophoresis in H-E buffer. To test this we carried out electrophoresis for 6 h of labelled *Bacillus subtilis* 23S and 16S RNA, KB-cell rRNA and *Rhynchosciara* rRNA in 1% agarose gel slabs in H-E buffer which included a poly(U)-containing glass-fibre filter at 5 mm from the slots in which the RNA samples were placed in the gels. We found that in no case was there any binding of radioactivity to the filters after electrophoresis.

Next we tested whether increasing amounts of synthetic [³H]poly(A) or *Rhynchosciara* salivary-gland ³H-labelled RNA would bind proportionally to the poly(U)-containing glass-fibre filter under the conditions of electrophoresis in H-E buffer. To this end, an experiment similar to the one described above was carried out, but in which various amounts of *Rhynchosciara* salivary-gland ³H-labelled RNA

Fig. 1. Diagrammatic representation of the two-dimensional electrophoresis method

(shown to contain poly(A)-containing RNA) or [^3H]poly(A) were placed in slots located at 5 mm from a poly(U)-containing glass-fibre filter, which was placed in the electrophoresis path. At the end of the run the portion of the glass-fibre filter in front of each slot was cut out and assayed for radioactivity. As indicated in Fig. 3, under the conditions used there is a direct proportionality between the amount of radioactivity placed in the slots and that bound to the

poly(U)-containing glass-fibre filter after the electrophoresis.

Application of the method

The results outlined in the previous section indicated that all the necessary conditions for the specific binding of poly(A)-containing RNA during electrophoresis at high ionic strength did hold. Thus we

Fig. 2. Electrophoresis of synthetic $[^3\text{H}]\text{poly(A)}$ and *Rhynchosciara* salivary-gland ^3H -labelled RNA under high ionic strength

Electrophoresis was performed in 1% agarose in H-E buffer (0.01 M-Tris-HCl, pH 7.4, 0.12 M-NaCl, 0.5% sodium dodecyl sulphate) at 10 mA/cm² for 6 h. ●, *Rhynchosciara* ^3H -labelled RNA; ■, $[^3\text{H}]\text{poly(A)}$; 3 h run. The gels were cut into 1 mm fractions, which were assayed for radioactivity.

proceeded to apply the two-dimensional electrophoresis as described in the Materials and Methods section to the determination of the profile of poly(A)-containing RNA in RNA samples from *Rhynchosciara* salivary glands. The results obtained are shown in Fig. 4. They indicate that *Rhynchosciara* salivary-gland mRNA is dominated by forms moving as 18S and 15 Speaks (the S values were calculated by the method of Spirin, 1963); there is also poly-disperse mRNA with slower mobility (up to 38S). If our method is reliable, essentially the same result mentioned above should be obtained if we were to carry out an electrophoresis of RNA from the same sample as that used in Fig. 4, which had been previously purified by oligo(dT)-cellulose. When we carried out this experiment, the profile obtained (as shown in Fig. 5), although similar to that in Fig. 4, shows more radioactivity in the slow-moving RNA forms. This might be due to the fact that there was a great deal of the 38S rRNA precursor in the original sample (see Fig. 4) and that the 38S rRNA contaminated the mRNA sample even after its passage through oligo(dT)-cellulose. This seems to be the case, since when this oligo(dT)-cellulose-purified sample was analysed by two-dimensional electrophoresis, the profile (Fig. 6) is essentially similar to that shown in Fig. 4. These results indicate that binding to poly(U)-containing filters under the conditions of our two-dimensional electrophoresis is highly reproducible and specific. This method has also been used successfully by Brentani *et al.* (R.

Fig. 3. Binding of increasing amounts of $[^3\text{H}]\text{poly(A)}$ and poly(A)-containing ^3H -labelled RNA from *Rhynchosciara* salivary glands on poly(U)-containing glass-fibre filters by electrophoresis at high ionic strength

In each case a simple electrophoresis was performed in 1% agarose in hybridization (H-E) buffer. The gel slabs contained slots of 10 mm $\times$ 0.3 mm $\times$ 1 mm near the edges connected to the negative pole. At 5 mm from the slots and parallel to them, a strip of poly(U)-containing glass-fibre filter was placed across the gel slab. After the run, which was for 6 h, the portion of the glass-fibre filter in front of each slot was cut out and counted for radioactivity. Electrophoresis was performed as described in Fig. 1. ●, *Rhynchosciara* ^3H -labelled RNA; ■, $[^3\text{H}]\text{poly(A)}$

Brentani, J. M. Salles & N. Marques, personal communication) for the determination of the electrophoretic profile of collagen mRNA, giving a single 23S peak as expected from the molecular weight of collagen (Wang *et al.*, 1975).

Discussion

In the present paper we describe a method which allows the determination of the electrophoretic profile of the poly(A)-containing species present in a very small RNA sample (less than 50 μg). To achieve this, electrophoresis is first performed in agarose (polyacrylamide-gel electrophoresis could be used at this step) to separate the various RNA species present in the sample. The poly(A)-containing species are then detected by binding to a poly(U)-containing glass-fibre filter under electrophoresis in high-ionic-strength buffer in a direction perpendicular to the first electrophoresis. The poly(U)-containing glass-fibre filter is placed in the path of migration of the RNA.

It is fundamental to the reliability of the method that all RNA species move sufficiently during the

Fig. 4. Determination of the electrophoretic profile of poly(A)-containing RNA from *Rhynchosciara* salivary glands by the two-dimensional electrophoresis method

Total RNA was extracted from the salivary glands of *Rhynchosciara* larvae at period III of the fourth instar (age approx. 49 days, when the animals are just starting to spin the communal cocoon). For details of the two-dimensional electrophoresis method see the Materials and Methods section. Essentially, two equal samples of the RNA are first electrophoresed in low-ionic-strength buffer (E buffer) with 0.2% sodium dodecyl sulphate. One of these samples is used for localization of the main RNA fractions by u.v. scanning and for the determination of the radioactivity profile of the total RNA; the other sample is used to determine the amount of RNA of each fraction (separated in the first electrophoresis) that is able to bind to a poly(U)-containing glass-fibre filter placed in the path of a second electrophoresis (which is carried out under hybridization conditions). ●, Electrophoretic profile of total ^3H -labelled RNA; ■, profile of the ^3H -labelled RNA bound to the poly(U)-containing glass-fibre filter.

second electrophoresis to reach the poly(U)-containing glass-fibre filter in which the poly(A)-containing species will be bound. It is also important that the binding capacity of the poly(U)-containing glass-fibre filter is never exceeded at a given point. This can be determined by a previous assay of the amount of poly(A) in the sample to be analysed. In our laboratory we usually use $20\mu\text{g}$ of total cell RNA for the analysis. This quantity could be considerably decreased if large enough amounts of radioactivity were present in the poly(A)-containing RNA in the sample.

Fig. 5. Electrophoretic profile of *Rhynchosciara* salivary-gland ^3H -labelled RNA purified by oligo(dT)-cellulose chromatography

The same RNA as that used for the experiment shown in Fig. 4 was adsorbed on an oligo(dT)-cellulose by the procedure of Aviv & Leder (1972). The column was washed with 0.5M-KCl-0.01M-Tris-HCl, pH 7.4, and the bound RNA was eluted with 0.01M-Tris-HCl, pH 7.4. The RNA fraction eluted from the column was precipitated with 2.5vol. of 70% ethanol in 0.2M-potassium acetate and then resuspended in E buffer with 0.2% sodium dodecyl sulphate. A sample of this RNA was electrophoresed in E buffer with 0.2% sodium dodecyl sulphate under exactly the same conditions as those described in Fig. 4.

The first electrophoresis is carried out at low ionic strength and thus associations are minimized. This electrophoresis in agarose is specially suited for high-molecular-weight mRNA species such as those which have been found in insect cells (Daneholt, 1972). The resolution of the method for low-molecular-weight RNA can be considerably improved if the first electrophoresis in agarose is replaced by an electrophoresis in acrylamide gels.

Our method has the reliability of Kates's procedure (Sheldon *et al.*, 1972) but is simple to carry out because the binding of all fractions is carried out in a single-step operation in the course of electrophoresis in 'binding buffer'.

Fig. 6. Determination by the two-dimensional electrophoresis method of the electrophoretic profile of poly(A)-containing RNA from *Rhynchosciara* salivary-gland RNA purified by oligo(dT)-cellulose column chromatography

A sample of the RNA purified by oligo(dT)-cellulose chromatography used for the experiment shown in Fig. 5 was used for the determination of the electrophoretic profile by two-dimensional electrophoresis. The first electrophoresis was run at low ionic strength exactly as described in Fig. 4 and then a second electrophoresis was carried out, perpendicularly to the first electrophoresis, under hybridizing conditions, as described in Fig. 4.

Since a labelled sample is required in our method, the determination of the presence of a given species in the profile is dependent on its turnover. This is a disadvantage in relation to both Fraser & Loening's (1973) method and that of Bishop *et al.* (1974), which allow a titration of the amount of a species independent of its turnover.

We thank Dr. R. R. Brentani for critical reading of the manuscript. We are grateful to Dr. T. Yamane for a gift of radioactive poly(A), to Dr. H. Westphal for gifts of oligo(dT)-cellulose and ³H-labelled rRNA from KB cells and to Dr. W. Colli for a gift of *B. subtilis* ³H-labelled rRNA. This work was supported by Grants 73/015 and 74/166 from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) under the BIOQ-FAPESP project. M. C. O. was a predoctoral fellow from FAPESP.

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