Determination of Polyadenylate-Rich Ribonucleic Acid in the Nucleus and in the Cytoplasm of Plasmacytoma Cells

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A number of parameters affecting the adsorption of rRNA and poly(A)-containing RNA to Millipore filters were investigated separately. Binding of both types of RNA to the filter was dependent on the concentration of RNA, pH and Mg^{2+} concentration of the reaction mixture. Both types of RNA bound to the filter optimally at slightly acid pH values. The binding of poly(A)-containing RNA to the filter exhibited a broad pH-dependence compared with that of rRNA. The ratio of poly(A)-rich RNA/rRNA retained by the filter was maximal between pH 7 and 8. The presence of 1 mM-EDTA or a high concentration of NaCl (over 0.5M) decreased the affinity of RNA for the filter. The amount of poly(A)-containing RNA in the nucleus and in the cytoplasm of a plasmacytoma cell line (MPC-11) labelled with [^{32}P]P_i was determined by the Millipore-filter technique under conditions that minimized contamination by rRNA. These data were compared with the estimations made by oligo(dT)-cellulose chromatography. The results obtained by these two methods were in good agreement for RNA labelled for short periods (up to 2 h). In long labelling and pulse-chase experiments, however, contamination of the filter by rRNA of increasing specific radioactivity in the cytoplasm gave an erroneous value for poly(A)-containing RNA by the Millipore-filter technique. Determinations made on the nuclear fraction by these two methods did not show significant variation in short- and long-term labelling experiments.

The presence of a poly(A) sequence at the 3' end of eukaryotic mRNA provides a simple method for the separation of this class of RNA from other RNA species. Several separation techniques based on the property of the poly(A) region of the molecule have been developed (Edmonds & Caramela, 1969; Kates, 1970; Lee *et al.*, 1971; Kitos *et al.*, 1972; Sheldon *et al.*, 1972; Brawerman *et al.*, 1972). These techniques can be classified into two main groups, one utilizing hydrogen bonding between the poly(A) moiety of mRNA and poly(U) or poly(dT) attached to Sepharose, cellulose or glass-fibre filters, and the other utilizing the ability of mRNA to bind to a variety of substances such as cellulose, nitrocellulose, methylated albumin, polystyrene etc. Of these techniques, oligo(dT)-cellulose column chromatography is the method of choice for the purification of mRNA for the following reasons. (1) A high degree of selectivity is obtained by hybridization. (2) RNA can be adsorbed to the column in the presence of small amounts of sodium dodecyl sulphate usually added to RNA samples to inhibit ribonuclease activity. (3) The oligo(dT) sequence attached to the cellulose, being fairly short (10–12 nucleotides), enhances the discrimination against weaker interactions involving poorly attached or looped-out sequences. (4) RNA molecules containing short

segments of poly(A) sequences are also bound effectively.

The Millipore-filter technique, being inexpensive and simple in operation, is preferred for the routine assay of labelled mRNA. These filters are pure nitrocellulose and do not contain bound poly(U) or poly(dT). RNA adsorbed to the filter can be counted for radioactivity directly. This technique, however, has the disadvantage of being less specific. RNA bound to the filter at controlled pH and ionic strength was not entirely free of rRNA (Brawerman *et al.*, 1972). We have investigated the applicability of the Millipore-filter technique for the measurement of mRNA and describe a number of parameters that influence the selectivity and efficiency of the binding reaction.

Experimental

Materials

NN'-Dicyclohexylcarbodi-imide was obtained from Fluka AG, Buchs SG, Switzerland. TMP (free acid) and sodium dodecyl sulphate were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Carrier-free [^{32}P]P_i was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All other chemicals were standard analytical grade.

Methods

RNA was isolated from a mouse plasmacytoma cell line, MPC-11, which was grown in suspension culture as reported by Birckbichler & Pryme (1973). Details of the labelling of the cells with [32 P]P_i and the isolation of nuclear and cytoplasmic fractions are described in earlier papers (Abraham *et al.*, 1973; Eikhom *et al.*, 1975). All glassware used for the isolation and storage of RNA preparations was heated at 200°C overnight. Sequential extraction of RNA in neutral and alkaline Tris buffer was carried out at 4°C, by a modification of the phenol-sodium dodecyl sulphate procedure (Brawerman *et al.*, 1972). The nuclear and cytoplasmic fractions in saline buffer (0.15M-NaCl, 5mM-MgCl₂, 20mM-Tris-HCl, pH7.5, 0.1mM-EDTA) were diluted with an equal volume of 0.1M-Tris-HCl (pH7.9). Sodium dodecyl sulphate was added to the diluted samples to a final concentration of 0.5% (w/v). The samples were then shaken gently with an equal volume of water-saturated phenol in the cold for 20min. The water phase (pH approx. 7.0) was separated by centrifugation at 10000g for 10min in the cold. The phenol phase was re-extracted with an equal volume of 0.1M-Tris-HCl, pH7.9, in the presence of 0.5% sodium dodecyl sulphate. The aqueous phase (pH7.9) was combined with the water phase from the first extraction. After removing most of the phenol by two ether extractions, RNA was precipitated with 2.5 vol. of ethanol in the presence of 0.2M-sodium acetate (pH5.1) and 5mM-MgCl₂. The RNA samples were free of P_i after three precipitations with ethanol.

Oligo(dT)-cellulose was prepared by the method of Gilham (1964), by using the *NN'*-dicyclohexylcarbodi-imide reaction for the polymerization of dTMP and the coupling of oligo(dT) to the cellulose. Portions (100mg) of oligo(dT)-cellulose in 10mM-Tris-HCl buffer (pH7.6) containing 0.5M-NaCl and 1mM-EDTA were packed into small columns (1ml disposable syringes). Each column was washed with at least 50ml of the buffer before use. RNA samples in 3ml of the above buffer, containing up to 50µg of RNA/ml, were applied to equilibrated columns. The columns were then washed with 3ml of the same buffer. The flow-through was collected together with the first wash (fraction 1). The material retained by the column was eluted in three steps by lowering the ionic strength. The first elution buffer contained 0.2M-Tris-HCl, pH7.6 (fraction 2); the second 0.1M-Tris-HCl, pH7.6 (fraction 3); and the third 0.01M-Tris-HCl, pH7.6 (fraction 4). Each buffer in addition contained 1mM-EDTA. The material eluted in each fraction was precipitated immediately with 2.5 vol. of ethanol in the presence of 0.2M-sodium acetate and was stored at -20°C. Used oligo(dT)-cellulose was regenerated by a quick wash with 0.1M-KOH.

Gel electrophoresis of RNA was performed on pre-swollen bisacrylamide-cross-linked 3.1% (w/v) polyacrylamide gels (1cm diam.) as previously described (Eikhom *et al.*, 1975).

The adsorption of RNA to Millipore filters was carried out essentially as described by Brawerman *et al.* (1972) in a buffer containing 0.5M-KCl, 1mM-MgCl₂ and 10mM-Tris-HCl (pH7.6). RNA samples in the above buffer were passed through pre-soaked filters at a rate of 0.4ml/min. Unless otherwise stated, the concentration of RNA samples was not above 50µg/ml. The filter was washed after the binding of RNA with at least 10ml of the same buffer and dried for scintillation counting. Radioactivity was determined in a Nuclear-Chicago scintillation counter, by using 7ml of toluene-2,5-diphenyloxazole-1,4-bis-(5-phenyloxazol-2-yl)benzene 'cocktail' (Eikhom *et al.*, 1975).

Results and Discussion

Effect of pH and salt concentration on the binding of rRNA and poly(A)-containing RNA to Millipore filters

Since the Millipore-filter technique is both simple and inexpensive, we investigated the selectivity and efficiency of this technique, by varying the pH, ionic strength and the cations present in the binding buffer. A non-selective adsorption of RNA to the Millipore filters was observed at slightly acid pH (Fig. 1). This pH effect was more pronounced for the rRNA. When 15µg of rRNA or poly(A)-rich RNA in 10ml of the binding buffer was passed through Millipore filters separately, the ratio of poly(A)-rich RNA/rRNA retained by the filter was in the range of 56 to 35 between pH7.0 and 8.0. Increasing the concentration of Mg²⁺ from 1 to 10mM did not influence the binding significantly, whereas the addition of 1mM-EDTA instead of MgCl₂ decreased the binding of the RNA samples (Table 1). In the EDTA-containing buffer, the optimum pH for the non-specific adsorption of RNA shifted more towards neutral (results not shown). At pH7.6 and in the presence of 1mM-MgCl₂, the percentage of RNA retained by the filter at various RNA concentrations increased with increasing salt concentrations of up to 0.5M-KCl. KCl concentrations between 0.3 and 0.5 were optimum for the binding at room temperature (Table 1). The percentage of RNA bound to the filter decreased from 18.0 to 14.8, when the KCl concentration was increased from 0.5 to 2.0M (Table 1).

Effect of RNA concentration on the binding of rRNA and poly(A)-containing RNA to Millipore filters

Purified cytoplasmic RNA labelled for 8h was first fractionated by chromatography on oligo(dT)-cellulose. Gel-electrophoresis profiles of RNA

Fig. 1. Effect of pH on the adsorption of rRNA and poly(A)-containing RNA to Millipore filters

Labelled rRNA [20 μ g of fraction 1 from oligo(dT)-cellulose column] and poly(A)-containing RNA [5 μ g of fraction 4 from oligo(dT)-cellulose column] obtained after chromatographic separation of total cytoplasmic RNA as described in the Experimental section, were adsorbed to Millipore filters separately by using standard conditions described in the Experimental section. The pH of the binding buffer was varied from 5 to 6.5 with 20mM-sodium acetate-acetic acid buffer, and from 7 to 9 with 20mM-Tris-HCl buffer. $\circ$, rRNA; $\bullet$, poly(A)-containing RNA.

fractions eluted from the column (Fig. 2) showed that fractions 1 and 2 consisted predominantly of 18S and 28S RNA species. A small percentage of the RNA applied to the column was eluted with buffer of intermediate ionic strength (fraction 3). This fraction contained traces of 18S and 28S RNA species. The last fraction (fraction 4) did not contain the rRNA species. The radioactive peak in the first fraction of Fig. 2(d) was mainly aggregated mRNA, since the RNA was not subjected to denaturing conditions. After rechromatography of fractions 1 and 4 on a second oligo(dT)-cellulose column, the RNA in each fraction was eluted at the expected salt concentration. It was therefore concluded that fraction 1 was free of poly(A)-containing RNA, and fraction 4 free of rRNA. It should be emphasized, however, that inability of an RNA sample to bind to an oligo(dT)-cellulose column need not always imply that this sample is free of poly(A)-containing RNA. Head-to-tail aggregation of poly(A)-containing mRNA by base-pairing between poly(A) and poly(U) regions can happen if the RNA samples are not diluted sufficiently to avoid such interaction.

Table 1. Effect of salt concentration and temperature on the binding of RNA to Millipore filters

Cytoplasmic RNA samples in buffers containing 0.01M-Tris-HCl (pH 7.6) and appropriate concentrations of KCl and MgCl₂ or EDTA were filtered through Millipore filters as described in the Experimental section. Unless otherwise stated, the binding was carried out at room temperature. RNA used in these experiments was isolated from cells labelled for 1 h with [³²P]P_i.

Conditions for binding			Radioactivity bound to filter (%)
KCl (mM)	MgCl ₂ (mM)	EDTA (mM)	
0	1	—	5.2
100	1	—	7.2
200	1	—	10.3
300	1	—	17.4
500	1	—	18.0
1000	1	—	16.0
2000	1	—	14.8
500	10	—	17.8
500	—	1	13.2
500(0°C)	1	—	15.0
100(0°C)	1	—	7.8

Table 2 shows the binding of rRNA and poly(A)-rich RNA to Millipore filters under standard binding conditions described in the Experimental section. Adsorption of both types of RNA to the filter was concentration-dependent. For a given RNA concentration, the filter bound poly(A)-rich RNA more effectively than rRNA, as expected. The fraction of RNA bound to the filter remained fairly constant on repeated passages of a single sample through the filter. However, repeated passages of the filtrate after dilution with the binding buffer increased the fraction of RNA retained by the filter. This effect was more obvious with rRNA. Denaturation or dilution of rRNA from a buffer of low ionic strength into a large volume of binding buffer, to give a low concentration of RNA, increased the binding considerably. Under these conditions a very significant fraction of rRNA was bound to the filter (Table 2).

When 150 μ g of a reconstituted mixture of rRNA and poly(A)-containing RNA in the ratio 50:1 (relative concentrations in the cytoplasm) was applied to the filter in 10ml of the binding buffer, roughly 77% (2.3 μ g) of the poly(A)-containing RNA and 1.5% of the rRNA (2.2 μ g) were retained (Table 2).

Determination of the amount of poly(A)-rich RNA by Millipore-filter technique and oligo(dT)-cellulose chromatography

After establishing conditions for a more specific and effective binding of poly(A)-containing RNA to Millipore filters, we checked the applicability of this

Fig. 2. Polyacrylamide-gel-electrophoresis profiles of RNA fractions eluted from an oligo(dT)-cellulose column

Total cytoplasmic RNA (40 μ g) isolated from cells labelled for 8 h with [32 P]P_i was fractionated on an oligo(dT)-cellulose column as described in the Experimental section. After chromatography, 40 μ g of unlabelled RNA was added to fractions 2, 3 and 4. No carrier RNA was added to fraction 1, which contained more than 90% of the RNA applied to the column. Each fraction was precipitated with 2.5 vol. of ethanol and dissolved in 0.5 ml of 10 mM-Tris-HCl buffer (pH 7.4) containing 1 mM-EDTA and 0.2% sodium dodecyl sulphate. Sample volumes of 0.1 ml of fractions 1 and 4 and 0.2 ml of fractions 2 and 3 were used for gel electrophoresis, as described by Eikhom *et al.* (1975). All fractions refer to RNA fractions eluted from an oligo(dT)-cellulose column as described in the Experimental section. (a) Fraction 1; (b) fraction 2; (c) fraction 3; (d) fraction 4.

Table 2. Dependence of the Millipore-filter binding reaction on RNA concentration

Various concentrations of labelled RNA in 10 ml of binding buffer were adsorbed on Millipore filters at room temperature, by using standard conditions described in the Experimental section. Poly(A)-containing RNA and rRNA used in these experiments were obtained by oligo(dT)-cellulose chromatography of the total cytoplasmic RNA as described in the Experimental section (fractions 4 and 1 respectively).

RNA sample	Amount of RNA applied to the filter		RNA retained by the filter	
	(μ g)	(c.p.m.)	(c.p.m.)	(%)
Total cytoplasmic RNA	2	2700	122	4.5
	5	6750	252	3.8
	20	27000	838	3.1
	200	270000	5950	2.2
rRNA	3	2950	109	3.7
	10	9840	185	1.9
	200	196800	1780	0.9
Two repeated passages of rRNA after fivefold dilution each time	3	2950	345	10.3
Poly(A)-rich RNA	2	8700	7620	88.0
	10	43500	27400	63.0
Mixture of unlabelled rRNA and labelled poly(A)-rich RNA (ratio 1:50)	150	2490	1920	77.0
Mixture of labelled rRNA and unlabelled poly(A)-rich RNA (ratio 1:50)	150	75600	1138	1.5
Denatured rRNA in 0.1 ml of Tris-HCl buffer, diluted to 100 ml with the binding buffer	1	7890	2360	30.0

Table 3. *Estimation of poly(A)-rich RNA by Millipore-filter technique and oligo(dT)-cellulose chromatography*

The amount of poly(A)-containing RNA in the nuclear and cytoplasmic RNA fractions isolated from cells labelled with [32 P] P_i was determined by Millipore-filter technique and oligo(dT)-cellulose chromatography. Conditions for growing cells, labelling of nucleic acids, extraction of nuclear and cytoplasmic RNA, binding to Millipore filters and oligo(dT)-cellulose chromatography were as described in the Experimental section. Equal amounts of each RNA sample were used in both methods.

Duration of labelling (h)	Duration of chase (h)	RNA preparation	Total RNA used		Radioactivity retained (% of total counts)	
			(E_{260} units)	(c.p.m.)	Millipore filter	Oligo(dT)-cellulose
1	—	Nuclear	0.3	7100	9.5	12
		Cytoplasmic	3.0	8700	16.0	19
2	—	Nuclear	0.4	12500	8.0	11
		Cytoplasmic	2.1	45300	14.0	15
8	—	Nuclear	0.3	31900	6.8	7.9
		Cytoplasmic	2.5	231800	8.9	6.2
24	—	Nuclear	0.3	36900	1.8	1.5
		Cytoplasmic	2.0	803700	4.0	1.5
1	8	Nuclear	0.2	21000	2.0	2.3
		Cytoplasmic	2.8	73700	6.3	4.4

technique for the measurement of mRNA in plasmacytoma cells, by comparison with the results obtained by oligo(dT)-cellulose chromatography. The results presented in Table 3 clearly show that although the values obtained by the two methods are comparable for short labelling periods, in long labelling and pulse-chase experiments the results obtained by these two methods differed significantly. This was more so for the cytoplasmic RNA fraction, with a net increase in labelled RNA that did not contain a poly(A) sequence. Millipore filters, in contrast with oligo(dT)-cellulose columns, do not bind RNA molecules possessing short stretches of poly(A) (Brawerman, 1973). It is also conceivable that poly(A) sequences in some of the rRNA species do not have the right configuration necessary for the binding to Millipore filters. Therefore the lower values obtained by the filter technique for the rRNA most probably reflect a difference in the conformation of the poly(A)-containing RNA species.

When the duration of labelling was increased, the percentage of radioactive cytoplasmic RNA retained by the Millipore filter was higher than the percentage bound to oligo(dT)-cellulose columns. Since the incorporation of radioactive P_i into rRNA increases in proportion to the duration of labelling, a slight contamination of the filter with rRNA of increasing specific radioactivity could explain the above discrepancy.

For short labelling periods of up to 2 h, contamination of the filter by rRNA does not cause any serious problem for the measurement of mRNA by this method. However, in pulse-chase experiments designed to measure the turnover rate of mRNA,

label in rRNA fractions can be significant at times owing to the metabolic stability of rRNA compared with mRNA.

Conditions for the adsorption of RNA to Millipore filters indicate that hydrogen bonding or ionic interactions within the RNA molecules are not responsible for the preferential binding of poly(A)-containing RNA species. Poly(A) has been shown to exist at neutral pH values as single-stranded helical structures stabilized by base stacking and containing practically no intramolecular or intermolecular hydrogen bonding (Brahms *et al.*, 1965). Therefore it seems reasonable to assume that an RNA conformation close to that of poly(A) at neutral pH is responsible for interaction with the filter. The extent of intramolecular base stacking of poly(A) molecules at neutral pH was shown to increase with increasing salt concentration and decreasing temperature (Brahms *et al.*, 1965). Conditions that favour RNA binding to filters, high salt concentration and low temperature (Brawerman *et al.*, 1972), also increase intramolecular base stacking of RNA molecules. Intermolecular interaction forming base-paired regions could destabilize the base-stacked molecules. Such a destabilization of the RNA molecules might decrease the base-stacking interaction with the aromatic rings on the Millipore filter. This would explain the concentration-dependence of the RNA binding to the Millipore filters. Since the amount of RNA retained by the filter was somewhat decreased in buffers containing more than 0.5M-KCl (Table 1), the requirement for the binding reaction, perhaps, is not simply an RNA conformation maximally stabilized by base stacking. It was reported that the

mechanism for the binding of poly(A)-containing RNA, and to a certain extent rRNA, to benzoylated cellulose involved base-stacking interaction between RNA and benzoylated cellulose (Roberts, 1974). Adsorption of RNA on cellulose and nitrocellulose requires polyaromatic lignins in these materials (Sullivan & Roberts, 1973).

We propose a base-stacking mechanism also for the binding of RNA to Millipore filters. Such a mechanism could explain the non-specific binding of rRNA and the dependence of the binding reaction on pH, ionic strength and RNA concentration. All natural single-stranded RNA molecules contain areas that are not base-paired to form double-stranded regions (Boedtker, 1967). In dilute RNA solutions, intermolecular interactions are considerably decreased, and the possibility for base-stacking interaction with the aromatic residues of the Millipore filter is increased.

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