URIDYLATE-RICH SEQUENCES IN RAPIDLY LABELLED RNA OF MAMMALIAN CELLS

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1. Introduction

Much interest has centred recently around the occurrence of adenylate-rich sequences in rapidly labelled RNA species. Such sequences have been detected in polyribosomal RNA, in messenger RNA [1–9] as well as in the heterogeneous nuclear RNA (HnRNA) fraction [1–4]. Another striking feature of HnRNA is its overall base composition. Despite being commonly referred to as "DNA-like", literature values [8, 10] reveal the proportion of uracil to be considerably higher (29 to 32%) than the proportion of adenine (24 to 26%). This has prompted the present examination of mammalian pulse-labelled RNA for the possible presence of uridylate-rich regions by virtue of formation of hybrids with polyriboadenylic acid (poly(rA)).

2. Materials and methods

BHK-21/C13 cells cultured in Eagle's medium supplemented with 10% (v/v) calf serum as monolayers in rotating 80 oz bottles were exposed to ^{3}H-uridine (specific activity 55 Ci/mmole) for various short lengths of time and the labelled RNA extracted using the hot phenol–detergent technique at pH 5.1 [11]. ^{3}H-stable cytoplasmic RNA species (i.e. tRNA, 5 S RNA and high molecular weight ribosomal RNA) from these cells grown in the presence of ^{3}H-uridine (specific activity 5.4 Ci/mmole) for 88 hr, were prepared by cold phenol extraction followed by Sephadex G-100 chromatography [11].

DNAase (electrophoretically purified) and RNAase were purchased from Sigma (London) Chemical Co. Ltd. Actinomycin D was from Calbiochem and polyriboadenylic acid (approx. 8.95), polyuridylic acid, polycytidylic acid, ^{3}H-polycytidylic acid and ^{3}H-polyuridylic acid were obtained from Miles Laboratories Inc. Cellulose ester membrane filters (25 mm diam., 0.22 μ pore size) were purchased from Millipore (UK) Ltd.

SSC is 150 mM NaCl–15 mM Na citrate pH 7.6 and KTM buffer is 500 mM KCl–10 mM Tris (pH 7.6)–1 mM $MgCl_2$.

3. Results

The high affinity of poly (rA) in high ionic strength buffer (KTM) for Millipore filters has recently been demonstrated [7]. Indeed RNA species containing poly(rA) sequences are known to be retained by these filters [12]. RNA from BHK-21/C13 cells labelled for various times with ^{3}H-uridine and extensively pretreated with deoxyribonuclease has been examined for its ability to bind to Millipore filters. The proportion of such molecules, presumed to contain poly(rA) sequences, increases during the first 45 min of labelling (fig. 1, solid circles) but thereafter declines slowly. Evidence that such species are fairly stable is provided in fig. 2 (solid circles). Actinomycin D addition to the culture medium results in a considerable loss of labelled RNA but the level of RNA species containing poly(rA) sequences remains constant.

Since poly(rA) is retained by Millipore filter it seemed reasonable to search for RNA species containing uridylate-rich regions by simply incubating the labelled RNA with added non-labelled poly(rA).

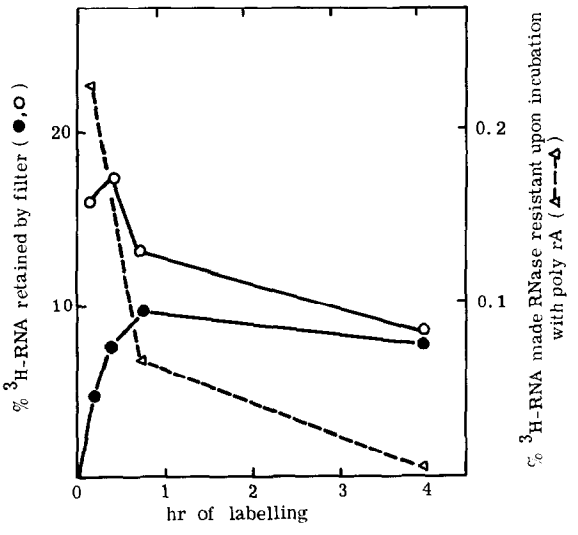

Fig. 1. Time course of formation of poly(rA) containing RNA's and RNA's containing uridylate-rich regions. Aliquots of ^{3}H-RNA (approx. 45 μg) from cells labelled for various times with ^{3}H-uridine were incubated in 1 ml of 0.1 M Tris (pH 7.6)–SSC with or without 10 μg poly(rA) for 60 min at 30° then filtered through Millipore filters as described by Brawerman et al. [12] after dilution with 15 ml KTM buffer. The filters were further washed with KTM buffer and after drying were assayed for radioactivity in toluene based scintillator fluid [11]. The % ^{3}H-RNA retained by the filters after incubation with poly(rA) is indicated by (○—○—○) whereas that retained after incubation in the absence of poly(rA) is shown by (●—●—●). A similar set of incubations was set up but after the incubation they were digested with RNAase (10 μg) for 30 min at room temp. before the addition of KTM buffer and filtration described above. (△---△---△) represents the % ^{3}H-RNA retained by the filter but which has been rendered RNAase-resistant during the incubation with poly(rA).

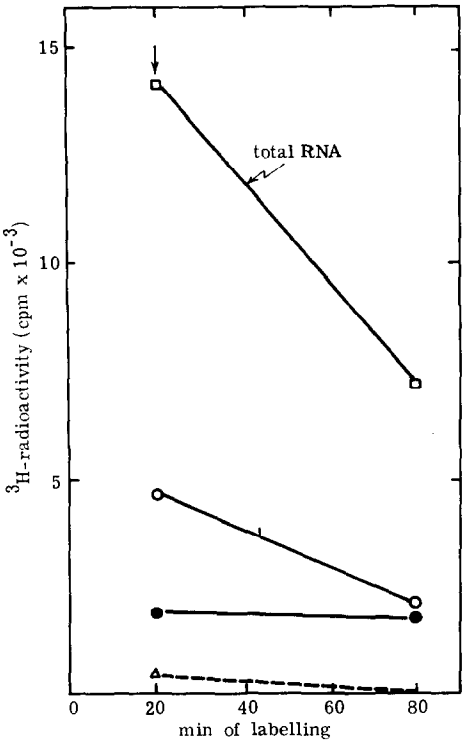

Fig. 2. Effect of actinomycin D. In this case ^{3}H-RNA was prepared from cells incubated for 20 min with ^{3}H-uridine and from cells incubated with ^{3}H-uridine for this period but then further treated with 4 μg actinomycin D/ml of medium for a further 60 min. Aliquots (approx. 45 μg) of these ^{3}H-RNAs were then incubated with and without poly(rA) exactly as described in fig. 1. The total level of radioactivity retained by the filters after incubation with poly(rA) (○—○), the total retained after incubation in the absence of poly(rA) (●—●), the level of radioactivity retained by the filters which had also been rendered RNAase-resistant during the incubation with poly(rA) (△---△).

Also shown (□—□) is the total level of radioactivity in each aliquot of RNA examined in the above context. The arrow indicates the addition of actinomycin D.

Any uridylate-rich region might be expected to hybridise with at least part of this added poly(rA) which would also serve to bind the hybrid to the filter. From fig. 1 (open circles) it can be seen for instance that prior incubation of the 12 min labelled RNA with 10 μg of poly(rA) in SSC at pH 7.6 for 1 hr at 30° leads to an increase in the level of ^{3}H-RNA retained by the filter. This increased retention of ^{3}H-RNA could be inhibited by including polyribouridylic acid as well as the poly(rA) in the preincubation mixture. Polyribocytidylic acid addition on the other hand was not inhibitory.

The increased level of ^{3}H-RNA retained by the filters after incubation with poly(rA) is, however, much less evident when the RNA isolated from cells labelled for slightly longer times is examined (e.g. 4 hr). Similarly, examination of the RNA from cells exposed to actinomycin D (see open circles, fig. 2) indicates a marked loss of the species that showed increased retention after incubation with poly(rA). In the case of the ^{3}H-labelled stable RNA's of BHK-21/C13 cell cytoplasm i.e. high molecular weight

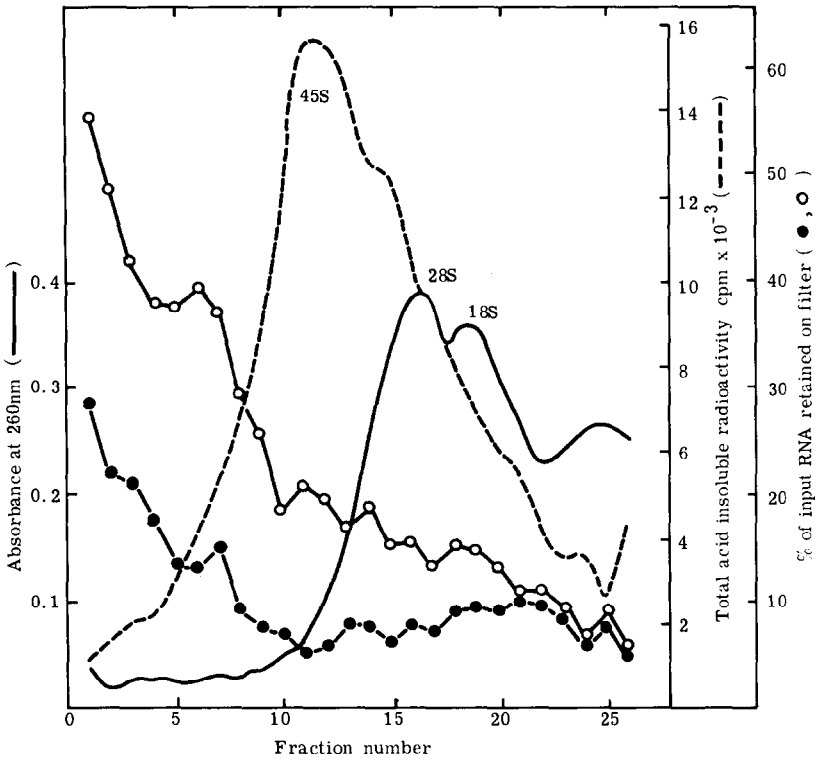

Fig. 3. Analysis of sucrose density gradient fractions of rapidly labelled RNA. A 0.3 ml sample of 20 min labelled ^{3}H-RNA was layered on a 4.6 ml 5–20% sucrose gradient containing 0.1 M NaCl, 0.02 M Tris (pH 7.6) for 90 min at 40,000 rpm in a Spinco SW 50L rotor. Fractions were collected into sterile water, their absorbance determined and the level of ^{3}H-radioactivity assayed in suitable aliquots. The remainder of each fraction was then divided and incubated as described in fig. 1 with and without poly(rA), and then filtered as already described through Millipore filters in KTM buffer. Absorbance at 260 nm (——); total ^{3}H-radioactivity in each fraction (------); % radioactivity retained by filter after incubation with poly(rA) (○—○); and % radioactivity retained by filter after incubation in the absence of poly(rA) (●—●).

ribosomal RNA, 5 S RNA and tRNA, no retention by the filters was observed, either before or after incubation with poly(rA).

Whether the increased retention of the rapidly labelled RNA was, as seemed likely, due to the formation of hybrids between the poly(rA) and apparently short-lived uridylate-rich regions in certain species of rapidly labelled RNA was tested as follows. Firstly the various ^{3}H-RNA preparations were incubated with poly(rA) as before, but then digested with RNAase (10 μg/ml) prior to filtration through Millipore filters in KTM buffer. The observed level of RNAase-resistant ^{3}H-radioactivity found to be retained by the filters due to the incubation with poly(rA) is shown in figs. 1 and 2 (open triangles). The formation of these RNAase-resistant complexes is optimal at around 30° and is complete in 60 min at the nucleic acid concentrations used. Once formed they appear quite stable and resistant to up to 50 μg/ml ribonuclease. Similar complexes could be formed and detected under these conditions with ^{3}H-polyribouridylic acid but not with ^{3}H-polyribocytidylic acid or ^{3}H-tRNA, ^{3}H-5 S RNA or ^{3}H-ribosomal RNA prepared from BHK-21/C13 cells.

Sucrose density gradient fractionation of RNA from cells labelled for 20 min revealed the RNA with the greatest proportion of poly(rA) containing species to sediment faster than 45 S as anticipated (fig. 3). This was also found to be the case for the RNA species presumed, by the above type of examination, to contain uridylate-rich regions. In addition however poly(rA) containing species also comprise a

significant proportion of the RNA molecules sedimenting between 28 S and 4 S.

4. Discussion

The data are all compatible with the hypothesis that at least some HnRNA species contain uridylate-rich sequences. These sequences, and possibly the RNA to which they are covalently attached, are somewhat unstable in contrast to the poly(rA) containing species. Like the poly(rA) sequences however they could be added as a post-transcriptional event. An enzyme activity capable of adding uridylate *as well as* adenylate units to the 3′-ends of RNA molecules has been detected in nuclear ribonucleoprotein particles [13]. Alternatively they could arise as the result of transcription from specific genomic sites. Mammalian cells certainly have polydeoxadenylate-rich sequences distributed throughout the genome [14] and these may have special significance in this context.

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